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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/87, A61K 47/87	A2	(11) International Publication Number: WO 96/21036
		(43) International Publication Date: 11 July 1996 (11.07.96)

(21) International Application Number: PCT/US95/17005

(22) International Filing Date: 26 December 1995 (26.12.95)

(30) Priority Data:
08/366,787 30 December 1994 (30.12.94) US

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: NUCLEIC ACID CONDENSING AGENTS WITH REDUCED IMMUNOGENICITY

(57) Abstract

Nucleic acid condensing agents with reduced immunogenicity are generated either by conjugation of polycations or by selection of basic amino acid regions from proteins. Conjugation involves a chemical linkage between a polyalkylene glycol, such as polyethylene glycol, or a polysaccharide, such as dextran, and a polycation. Additionally, gene delivery vehicles, such as viral vectors, may be conjugated with polyalkylene glycol or polysaccharide, to reduce their immunogenicity. Basic amino acid regions of proteins are identified by isoelectric point, and amino acid composition. These condensing agents are complexed with nucleic acids and used to deliver genes to cells. Immunogenicity is assessed by whether neutralizing antibody is induced and by whether a serum componentinactivates the complexes.

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NUCLEIC ACID CONDENSING AGENTS WITH REDUCED IMMUNOGENICITY

Technical Field

This application relates to nucleic acid condensing agents, and in particular, to agents with reduced immunogenicity that are useful for gene transfer *in vivo*.

Background of the Invention

Successful gene transfer into animals or humans requires a gene delivery vehicle that can transport genetic information into target cells for expression of desired therapeutic proteins. In general, such gene delivery vehicles are either virus vectors, notably based on retroviruses, adenoviruses and vaccinia viruses, or non-viral vectors, which utilize a physical gene transfer mechanism.

Nucleic acids, typically DNA, delivered by a physical gene transfer mechanism is usually delivered by a receptor-mediated endocytosis pathway, a cellular mechanism which internalizes specific macromolecules. In general, complexes designed to be delivered in this fashion contain DNA encoding the gene of interest and a polycation which acts as a DNA binding domain and both neutralizes the charge on DNA and condenses the DNA. Condensation facilitates entry of DNA into cell vesicle systems by simulating a macromolecular structure. Optionally, the complex includes a ligand which directs the complex to particular cells expressing the ligand-binding partner, and an endosomolytic agent, which serves to cause disruption of the endosome containing the complex.

In vivo delivery of DNA by physical gene transfer has resulted in the successful expression of human serum albumin in rats (Wu et al., *J. Biol. Chem.* *266*:14338, 1991) and luciferase gene to the airway epithelium of cotton rats (Gau et al., *Hum. Gene Ther.* *4*:17-24, 1993). However, the efficiency of gene transfer in these systems was less than predicted from *in vitro* tests. Subsequently, it has been shown that such DNA complexes are unstable *in vivo*. Specifically, the polylysine component used in these complexes is thought to be immunogenic, eliciting a humoral immune response, and may additionally or alternatively be inactivated by a serum component, probably a protein in the complement pathway. This instability reduces the overall efficiency of gene transfer *in vivo*.

The other commonly employed gene transfer mechanism, viral vectors, are typically foreign agents to the host. The proteins of the viral capsids may be recognized as

foreign and an immune response is generated. Thus, the usefulness of these vectors for multiple administrations is limited. Initial administration may also elicit an undesirable inflammatory response if the individual had prior exposure to wild-type virus (*See*, Jolly, *Cancer Gene Therapy 1*:51, 1994).

In view of the problems associated with current gene delivery mechanisms, there is a compelling need for polycations which are equally or more effective and are not associated with the disadvantage of being immunogenic. The present invention provides compositions and methods for the use of compounds conjugated to polycations and gene delivery vehicles that reduce immunogenicity and novel condensing agents with reduced immunogenicity, as well as other, related advantages.

Summary of the Invention

The present invention generally provides nucleic acid condensing agents in which the nucleic acid condensing agent, in combination with nucleic acids, exhibits low or negligible immunogenicity. In one aspect, nucleic acid condensing agents are provided, comprising a polycation chemically conjugated with polyalkylene glycol. In a preferred embodiment, the polyalkylene glycol is polyethylene glycol. It is preferred that the polyethylene glycol has a molecular weight of 200 to 10,000. In a second aspect, nucleic acids are condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with polysaccharide. In a preferred embodiment of this aspect, the polysaccharide is dextran. In a further embodiment, the dextran has a molecular weight in the range 1,000 to 90,000. In embodiments of the invention, the polycation is selected from the group consisting of polylysine, protamines, histones, spermine, spermidine, polyornithine, polyarginine, and putrescine. In other embodiments, the nucleic acids further comprise a pharmaceutically acceptable carrier. In yet other embodiments, a ligand capable of targeting the nucleic acids to a selected cell type is included. Representative examples of such ligands include transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

In another aspect, gene delivery vehicles are provided that are chemically conjugated with polyalkylene glycol, such that the conjugated product exhibits low immunogenicity. In one embodiment of this aspect, the polyalkylene glycol is polyethylene glycol. It is preferred that the polyethylene glycol has a molecular weight ranging from 200 to 10,000. In a related aspect, gene delivery vehicles are chemically conjugated with a polysaccharide, such that the conjugated product exhibits low immunogenicity. In one embodiment, the polysaccharide is dextran. In a further embodiment, dextran has a

molecular weight ranging from 1,000 to 90,000. In other embodiments of the invention, the gene delivery vehicle further comprises a pharmaceutically acceptable carrier. In yet other embodiments, the gene delivery vehicle further comprises a ligand capable of targeting the gene delivery vehicle to a selected cell type.

Within still other aspects of the invention, nucleic acids condensed with a nucleic acid condensing agent comprising a basic amino acid region of at least 50 amino acids, in which the region contains at least 40% basic amino acids, has a predicted isoelectric point of at least 9, and contains not greater than 5% acidic amino acids are provided. In addition, the combination of nucleic acid condensing agent and nucleic acids exhibits low immunogenicity. The basic amino acid region may be derived from a protein, such as human serum albumin, histone, DNA binding proteins, protamines and/or non-histone chromosomal proteins. In one embodiment of this aspect, a plurality or multiplicity of basic amino acid regions are linked in a tandem array. In a related embodiment, the array contains between 1-10 basic amino acid regions. In other embodiments of the invention, the nucleic acids further comprise a pharmaceutically acceptable carrier and a ligand capable of targeting the nucleic acids to a selected cell type.

In another aspect, methods of gene transfer in a patient are provided, comprising the step of administering to a patient nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated either with polyalkylene glycol or polysaccharide, a gene delivery vehicle chemically conjugated with polyalkylene glycol or polysaccharide, nucleic acids condensed with a nucleic acid condensing agent comprising a basic amino acid region. These nucleic acids or gene delivery vehicles are additionally in a carrier pharmaceutically acceptable to a patient. In embodiments of this method, a ligand, which is capable of targeting the nucleic acids to a selected cell type is also provided. In yet other embodiments, the nucleic acids encode a protein, are transcribed into other nucleic acids, or themselves are capable of stimulating an immune response, suppressing an immune response, encoding a prodrug, encoding a cytokine, which is administered to a tumor, or capable of constitutive production of proteins.

These and other aspects of the invention will become evident upon reference upon the following detailed description. In addition, various references are set forth below which describe in more detail certain procedures or compositions. Each of these references are incorporated herein by reference in their entirety as if each were individually noted for incorporation.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Nucleic acid condensing agent" refers to a compound, natural or synthetically produced, which when combined with nucleic acids causes it to assume a condensed structure relative to uncomplexed nucleic acids. Condensation of DNA molecules to a size that may be internalized by coated pit structures present on cell surface membranes is preferable.

"Polycation" refers generally to a polymer of positively-charged single units, although some non-positively charged units may be present in the polymer. Preferably, for polycations that are polypeptides, they should have a predicted isoelectric point of at least 9 and contain at least 40% basic residues (generally, lysine plus arginine). Additionally, there should not be greater than 5% acidic residues and preferably none. Examples of polycations include polylysine, polyarginine, polyornithine, spermine, spermidine, putrescine, and protamine. For polycations composed of amino acids, either the L- or D-forms may be used. Basic amino acids include lysine, arginine, amino acid analogues such as ornithine and canaline, modifications of basic amino acids, such as homoarginine, and modifications of other amino acids such as to carry a positive charge, such as guanidinovalinate, and aminoethylcysteine. As a general rule, a basic amino acid has a pK value for the side chain of at least 7.5.

"Basic amino acid region" refers to domains of 50 to 300 amino acids in length that have a predicted isoelectric point of at least 9, and at least 40% basic residues. In most proteins, these residues are generally arginine and lysine. In addition, there should be not greater than 5% acidic amino acid residues. Basic amino acid regions may be derived from known proteins, including DNA binding proteins, such as histones and transcription factors, coat proteins from DNA viruses, or be synthetically derived.

"Immunogenicity" refers to the ability of a given molecule or a determinant thereof to induce the generation of antibodies with binding capacity to the molecule upon administration *in vivo*, to induce a cytotoxic response, activate the complement system, induce allergic reactions, and the like. An immune response may be measured by assays that determine the level of specific antibodies in serum, by assays that determine the presence of a serum component that inactivates the condensing agent/nucleic acid complex or conjugated gene delivery vehicle, or by other assays that measure a specific component or activity of the immune system. As discussed in more detail below, low immunogenicity may be established by these assays. The terms "low immunogenicity," "reduced immunogenicity," "lowered immunogenicity" or similar terms are intended to be equivalent terms. Because of the low immunogenicity of the nucleic acid/condensing agent complexes

of this invention, these complexes can be administered multiple times to the same host without eliciting unfavorable immune responses.

"Polyalkylene glycols" refer to 2 or 3 carbon polymers of glycols. Two carbon polyalkylenes include polyethylene glycol (PEG) of various molecular weights, and its derivatives, such as polysorbate, polyoxyethylene sorbitan monolaurate, and polyethylene glycol-*p*-isooctylphenyl ether. Three carbon polyalkylenes include polypropylene glycol and its derivatives.

"Polysaccharides" refer to polymers of three or more monomeric sugars. These sugars include D-glucose, D-mannose, D-fructose, D-galactose, L-galactose, D-xylose and D-arabinose. In addition, derivative monosaccharides may also be polymerized. Such derivatives include D-glucosamine, D-glucuronic acid, N-acetylmuramic acid, and N-acetyl neuraminic acid. Polymers of these monosaccharides may be composed of one type of saccharide or multiple types of saccharides and may be various molecular weights. Naturally occurring polysaccharides that may be used within the scope of this invention include dextrans, α-amylose, amylopectin, amylase-modified versions of polysaccharides, fructans, mannans, xylans, and arabinans.

"Nucleic acid" refers to DNA, RNA, analogues thereof, peptide-nucleic acids, and DNA or RNA with non-phosphate containing nucleotides. Additionally, these nucleic acids may be single-stranded, double-stranded, or chimeric single- or double-stranded molecules.

"Gene delivery vehicle" refers to an assembly which is capable of directing the expression of sequence(s) or gene(s) of interest. The gene delivery vehicle will generally include promoter elements and may include a signal that directs polyadenylation. In addition, the gene delivery vehicle includes a sequence which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. The gene delivery vehicle may also include a selectable marker such as *neo*, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the gene delivery vehicle is a retrovirus, a packaging signal and long terminal repeats (LTRs) appropriate to the retrovirus used will be included (if these are not already present). Further, if the gene delivery vehicle is a Sindbis virus, the vehicle RNA will include a 5' sequence which is capable of initiating transcription, as well as sequences which, when expressed, code for biologically active Sindbis non-structural proteins (*i.e.*, NS1, NS2, NS3, and NS4). In addition, the Sindbis gene delivery vehicle should include a Sindbis RNA polymerase recognition sequence, and a viral junction region, which may, in certain embodiments, be modified in order to either prevent or inhibit viral transcription of the subgenomic fragment. The gene delivery vehicle may also include nucleic acid molecule(s) which are of a size sufficient to allow production

of viable virus, as well as one or more restriction sites. When the Sindbis vector genome is transfected as a DNA molecule to give a eucaryotic layered vector system (ELVS), it should additionally include a 5' RNA polymerase II promoter which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination and splice recognition.

CONDENSING AGENTS

Nucleic acid condensing agents facilitate the transport of DNA or other nucleic acids into a cell. Such agents should not themselves be immunogenic or the efficiency of gene transfer will be diminished. This invention provides nucleic acid condensing agents of low immunogenicity comprising polycations conjugated with molecules and basic amino acid regions generally derived from proteins native to the species which is the target of gene transfer.

In general, to obtain a condensing agent within the context of this invention, the following strategy is employed. First, the condensing agent is prepared. Polycations are chemically conjugated with polyalkylene glycols, polysaccharides, derivatives, or similar compounds. A second type of condensing agent, novel condensing agents, comprising basic amino acid regions, are identified by interrogation of protein databases. The candidate regions are then chemically or biologically synthesized.

1. Preparation of polycations

Homopolymers of polylysine may be obtained from Sigma (St. Louis, MO) (*e.g.*, product #2636). Similarly, other homopolymers of several other polycations having various average lengths, such as polyarginine and polyornithine, may also be obtained from Sigma. Histone proteins, including H1, H3, and H4, may be obtained from Boehringer Mannheim (Indianapolis, Indiana). Spermine or spermidine, as well as protamine chloride, may be obtained from Sigma.

Preparation of these polycations and those which are derived from basic amino acid regions of a suitably identified protein may be synthesized by standard chemical techniques in lengths up to approximately 50 amino acids. Alternatively, the corresponding region of the gene may be isolated by conventional techniques such as PCR amplification using primers containing restriction sites chosen for convenience. Such amplified fragments may then be cloned and expressed in any of a variety of commercially available expression vectors designed for constitutive or transient expression in bacteria, yeast, or other eukaryotic cells.

As one such example, an amplified nucleic acid of interest is cloned into a bacterial expression system and the resulting polypeptide is expressed and purified

following the manufacturer's protocols. A complete bacterial high level expression and purification system known as Glutathione S-Transferase (GST) Gene Fusion System™ may be obtained from Pharmacia, Piscataway, NJ. Briefly, amplified DNA is cloned into the polylinker of one of the PGEX vectors from Pharmacia. The choice of PGEX vector is based on maintaining the desired reading frame for the cloned fragment and choosing either a factor Xa or thrombin protease recognition sequence as desired for cleavage during purification to remove the GST domain from the fusion protein. The vectors supply all necessary transcription and translation initiation sequences for high level inducible expression of either single or multiple units, obtained by simple sub-cloning procedures, of the desired basic domain. Fusion proteins containing the 26 kDa GST domain from *S. japonicum*, are purified from bacterial lysates by using the Bulk or RediPak GST Purification modules from Pharmacia. Cleavage of the desired amino acid domains from the fusion protein is achieved using a site specific protease, factor Xa or thrombin depending on the specific pGEX vector. All steps in expression and protein purification follow the manufacturer's protocols. Alternatively many other commercially available integrated expression/purification systems such as the "Flag" system from Kodak or other equivalent expression vector system may be used.

2. Conjugation of condensing agents

This invention provides polycations, useful as nucleic acid condensing agents, that have low immunogenicity. Polycations within the context of this invention include polylysine, polyarginine, histones, protamines, spermidine, spermine, and other highly basic proteins or polypeptides. In one embodiment, the polycation is chemically conjugated with one or more polyalkylene glycol. In one aspect of the invention, the polyalkylene glycol is polyethylene glycol. In another embodiment, the polycation is conjugated with one or more polysaccharides. Briefly, chemical conjugation involves forming a covalent linkage between the polycation and the polyalkylene glycol or polysaccharide. The conjugation may be performed either prior to or following condensation of the nucleic acid with the polycation. If conjugation is performed after condensation, other components discussed below, such as ligand molecules, may be present during the conjugation procedure. Many suitable methods for forming the linkage may be determined by one skilled in the art given the disclosure provided herein. In general, these methods first prepare the polyalkylene glycol or polysaccharide for coupling by creating an active group in place of a terminal OH group. A preferred reactive group for polyalkylene glycols is N-hydroxy succinimide. A preferred reactive group for polysaccharides is an aldehyde, which is present on some natural sugars or may be generated by chemical

oxidation. It will be recognized, however, that other reactive groups may be prepared by well-known chemical synthesis methods and used within the context of this invention.

As discussed above, in one embodiment, polycations are chemically conjugated with polyalkylene glycols. One example of such a polyalkylene glycol is polyethylene glycol (PEG). As an illustration of the invention, a model synthesis of conjugating PEG to polylysine is provided. Those in the art will recognize that other polyalkylene glycols and polycations, as well as alternative syntheses may readily be substituted. Briefly, the synthesis procedure first converts methoxy-PEG into a form amenable for conjugation, such as methoxypolyethylene glycol N-hydroxysuccinimidyl glutarate. Methoxy-PEG is conveniently used because it has only one hydroxyl group available for substitution. This compound may be prepared by the following procedure. PEG is first dissolved in toluene and distilled. The distilled PEG is reacted with glutaric anhydride in dicycloethane and dry pyridine to form PEG-glutarate. Following refluxing under nitrogen gas for three days, the mixture is filtered, and solvent is evaporated. The residue is dissolved in water and washed with diethyl ether. PEG-glutarate is then extracted from the water phase by chloroform washes. The chloroform is subsequently evaporated. PEG-glutarate is then dissolved in dimethylformamide and mixed with dicyclohexylcarbodiimide and N-hydroxysuccinimide in dimethylformamide and stirred vigorously. Upon the addition of benzene, methoxypolyethylene glycol N-hydroxysuccinimidyl glutarate (NHS-PEG) is precipitated by the dropwise addition of petroleum ether at 0°C. The precipitate is collected on a sintered glass filter, dissolved in benzene, and reprecipitated with petroleum ether. NHS-PEG may be stored at -20°C in a desiccator until use.

NHS-PEG may then be conjugated to the free ε-amino groups of polylysine essentially as follows. Briefly, NHS-PEG and polylysine are stirred together at a predetermined ratio for 30 minutes. Any remaining activated ester is removed by reaction with an excess of ε-aminocaproic acid. Unbound NHS-PEG is removed from the reaction mixture by size chromatography. The degree of amino group modification of the polycation may be determined by measuring the number of free amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) (Synder and Sobocinski, *Anal. Biochem. 64*:284-288, 1975) or NMR spectroscopy. The attachment of PEG to the polycation may also be confirmed by SDS-PAGE analysis or other suitable test. The optimum amount of PEG conjugation may be determined by the following two tests. The first test is to perform *in vitro* gene transfer and establish a ratio that reduces transfer efficiency of a reporter plasmid by about ten-fold. If this is not readily obtainable, a ratio near the maximum level of conjugation obtainable is chosen. Second, several conjugation ratios from zero to the near maximum ratio or the ratio that gives a ten-fold reduction in efficiency from the peak are

tested in the immunogenicity test as described below. The preferred ratio is that which gives a statistically significant reduction in immunogenicity as compared to an unconjugated polycation.

As set forth above, in a separate aspect, polysaccharides may be conjugated to polycations to reduce their immunogenicity. Although many procedures well known in the art may be used to prepare a polysaccharide for conjugation, one such procedure is provided below for illustrative purposes, wherein polysaccharides are conjugated to polycations following oxidation with $NaIO_4$. Briefly, polysaccharide is dissolved in sodium borate buffer containing $NaIO_4$ and stirred in the dark at room temperature for a day (Bobbit, *Adv. Carbohydrate Chem. 11*:1, 1956). The reaction is terminated by the addition of a molar excess of ethylene glycol. Oxidized polysaccharide is dialyzed extensively against distilled water and subsequently lyophilized. Subsequent conjugation of oxidized polysaccharides to polycations is performed as follows. A solution of oxidized polysaccharide is added at a predetermined ratio to a solution of polycation in sodium phosphate buffer. A solution of $NaBH_3CN$ in the same buffer is added at a large molar excess relative to polysaccharide, and the solution is stirred at room temperature for two hours. At the end of the incubation, dextran-conjugated polycation may be separated from unreacted polysaccharide by gel filtration. A relative aldehyde content of oxidized polysaccharide may be determined by an anthrone assay (Fagnani et al., *Cancer Res. 50*:3638-3645, 1990) or other suitable assay. Any free aldehyde groups remaining on the polycation dextran conjugate can be detrimental to the quality of the final product because these moieties can lead to the formation of undesirable high molecular weight aggregates, which tend to precipitate out of solution with time. To avoid this, the free aldehyde groups are reduced with a reducing agent, preferably $NaBH_4$. The extent of residual activation of oxidized dextran bound to the polycation may be determined by an anthrone/H_2SO_4 method (Fagnani, *supra*). An amount of $NaBH_4$ corresponding to the $NaBH_4$:oxidized dextran molar ratio producing greater than 80% reduction in color development is sufficient to adequately treat the polycation-dextran conjugates. Polysaccharide-conjugated polycations are then extensively dialyzed against sodium phosphate buffer. The optimal ratio of polysaccharide to polycation will depend upon the polycation used. The optimal ratio may be determined as described above.

Polysaccharides suitable for oxidation and subsequent conjugation to a polycation include homopolymers or heteropolymers of D-glucose, D-mannose, D-galactose, L-galactose, D-xylose, D-arabinose, D-glucosamine, D-glucoronic acid, N-acetyl-muramic acid and N-acetyl neuraminic acid. Natural polysaccharides that may be used include dextran, alpha-amylose, amylopectin, amylase-modified polysaccharides, fructans, such as insulin, mannans, xylans, and arabinans. Derivatives of these

polysaccharides such as carboxy methylcellulose may also be used. Dextran is a preferred polysaccharide because of its widespread use as a plasma substitute in current clinical practice.

Alternatively, the compound to be conjugated may be a synthetic polymer, other than PEG. Suitable polymers include homopolymers and copolymers which have either terminal or pendant functional groups for coupling to a polycation. A polymer's pendant functional groups results from a functional group present in the monomer from which the polymer is derived. Suitable terminal or pendant functional groups include any functional group which is or may be converted to a reactive functional group for coupling to a polycation.

A homopolymer is a polymer composed of a single repeating unit and is derived from the polymerization of a single monomer. For example, polymerization of vinyl alcohol yields polyvinyl alcohol, a homopolymer consisting of a carbon backbone (*i.e.*, repeating methylene groups) and pendant hydroxy groups. In the practice of the present invention, the pendant hydroxy groups may be further functionalized by standard organic chemical synthetic techniques to provide appropriate functional groups for coupling to a polycation. For example, acylation with succinic (or glutaric) anhydride or alkylation with a 2-haloacetic acid derivative of the hydroxy groups of polyvinyl alcohol provide polymers with pendant carboxylic acid functional groups. The carboxylic acid groups of these polymers may be converted directly to reactive esters such as N-hydroxy succinimide esters for coupling. Polyacrylic acid is an example of another suitable homopolymer consisting of a carbon backbone and pendant carboxylic acid groups.

Copolymers are also suitable as molecules for conjugation. A copolymer is a polymer composed of more than a single repeating unit and is derived from more than one monomer. For example, copolymerization of two different monomers (*i.e.*, comonomers) such as ethylene and maleic anhydride yields an ethylene/maleic anhydride copolymer consisting of a carbon backbone and pendant functional groups (*i.e.*, anhydride groups). Such a copolymer may be coupled to a polycation through reaction with the pendant anhydride functional groups.

In a similar manner, gene delivery vehicles may be conjugated with polyalkylene glycol or polysaccharide. In general, conjugation is performed in similar manner to that described above. A reactive group is synthesized for polyalkylene glycol, preferably a NHS group, and for polysaccharide, preferably an aldehyde. Reaction conditions may need to be modified in a routine, minor fashion depending on the virus undergoing coupling.

As is explained above, gene delivery vehicles including retroviral vector particles can be covalently modified for the purpose of reducing their immunogenicity with retention of significant biological activity. In addition, they can be derivatized via conjugation with specific tissue-targeting molecules to alter the targeting capabilities of these vectors. A variety of cross-linking agents can be used to derivatize proteins including heterobifunctional, homobifunctional, and photoreactive cross-linkers.

Heterobifunctional cross-linkers contain two reactive groups: a primary amine reactive group, usually N-Hydroxy-Succinimide (NHS) or its water-soluble derivative, sulfo-NHS, which reacts with primary amines such as the e-amino groups of lysine of proteins. The second reactive specificity of heterobifunctional cross-linkers is a thiol-reactive group, such as maleimides, halogens or pyridyl disulfides. By reacting with two distinct specificities, heterobifunctional cross-linking agents allow the linkage of two different proteins with significant reduction of unwanted side reactions like homoprotein polymers. Heterobifunctional cross-linking agents have been extensively used to covalently cross-link proteins to proteins, proteins to polymers, or polymers to polymers. A third component of a heterobifunctional cross-linking agent is the spacer arm, which connects the two reactive groups. The purpose of the spacer arm is primarily for steric hinderance and to provide added stability to the reactive groups of the cross-linking agent. Cross-linkers have spacer arms of varying lengths, as longer arms are at times required to effectively span the distance between two complex biomolecules and allow the reaction to take place.

Homobifunctional cross-linking agents possess two copies of the same functional reactive groups at both ends of a spacer arm. A majority of currently available homobifunctional cross-linkers are derivatives of either NHS or Sulfo-NHS esters while others are cross-linkers that do not contain NHS esters. The reaction conditions for NHS-based homobifunctional cross-linking agents are similar to the primary amine reaction of heterobifunctional cross-linkers. The NHS group reacts with primary amines such as the epsilon amino group of lysines or the N-terminus amine to form a stable amide bond with the release of N-hydroxy-succinimide as a by-product. This reaction is favored by neutral or alkaline pHs, where the primary amines are not protonated. They react by nucleophylic attack on NHS and by concentrated protein solutions, which favor the acylation reaction and reduce the inactivation of NHS by hydrolysis. By choosing a proper molar ratio of cross-linker to amine group, the extent of cross-linking can be varied to a desirable level.

Photoreactive heterobifunctional cross-linkers contain two different reactive sites, allowing the sequential conjugation of proteins with little or no polymerization or self-conjugation. The first step comprises the chemical linkage with the first protein to be conjugated. The linkage to the second protein then takes place under UV irradiation (Ji,

T. H. *Biochem Biophys Acta* 559:39, 1979). The photoactivation can be done by either exposing the reaction solution to direct light for 5-10 minutes or to 5-10 bright flashes. Cross-linkers containing phenyl azide require photoactivation in the 265-275 nm range, while those containing nitro-phenyl azyde achieve optimal photolysis around 320-350 nm, which limits damage to biomolecules due to radiation (Schrock, A.K. and Schuster, G.B.J. *Am. Chem. Soc.* 106:5228, 1984).

Gene delivery vehicles including retroviral vectors can be derivatized with water soluble polymers to generate conjugates with the retention of biological properties and little or no immunogenicity. Examples of such polymers include, but are not limited to, polyethylene glycol (Peg) derivatives, dextrans of various molecular weights, ethylene-maleic anhydrate copolymers, polyvinyl alcohols and others. Alternative derivatizations of retroviral vectors with water soluble polymers include; Peg-vinylsulfone, Peg-orthopyridyl-disulfide (OPSS-PEG), Peg-N-hydroxy-succinamide (PEG-NHS), Peg-Tresylate, Peg-allyl ether-maleic anhydrate copolymer, and Peg-biotin.

For example, Peg-maleimide can be used to generate biologically active Peg-protein conjugates (see Goodson, R.J. and Katre, N.V., *Biotechnology* 8:343, 1990; Kogan, T.P., *Synthetic Comm* 22:2417, 1992). Since this reaction requires free -SH groups, the protein to be derivatized may have to be treated with reducing agents in the case a free sulfhydryl is not available. Other techniques know to those of skill in the art can also be used to generate free -SH groups. For example, a free -SH group can be genetically engineered in a non-functional domain of the protein of interest to prevent potential reduction of biological activity resulting from the use of reducing agents. Retroviral vector preparations can be treated with optimal amounts of a reducing agent to generate free -SH groups under conditions that do not significantly affect vector activity. Ellman's reagent can be used to calculate the number of available sulfhydryls (Ellman, G.L., *Arch. Biochem. Biophys* 74:443, 1958).

Peg-maleimide is typically added to a solution of vector particles at molar ratios of 10-50 and a buffer pH of between 6.5 and 7.0. After incubation, Peg-derivatized vector particles can be purified from the reaction mixture using standard techniques such as gel filtration with Sephacryl S-400. Since derivatization reactions often proceed as a function of protein-to-polymer molar ratios, it may be useful to estimate the molar concentration of the candidate retroviral vector. This can be done, for example, by determining the number of vector particles mixed with polystyrene latex particles per unit volume by electron microscopy. In this procedure, the total number of vectors in a given preparation can be calculated based on the number of latex particles per unit of volume. Based on an estimated molecular weight of 200 million, the molar concentration of the vector can then be determined from the estimated molecular weight.. From this, the

relative concentration of specific vector proteins, can be estimated based on the known number of copies per vector particle. Alternatively, the mass of the capsid p30 and related proteins can be determined by Western blots or ELISA in comparison to a standard. The number of particles can be calculated assuming 3×10^3 of these molecules exist per particle. If the vector is formulated in a carrier protein such as human serum albumin (HSA), the molar ratio can be taken to be that of the carrier protein as it will also be modified.

The biological activity of the modified retroviral vectors can be determined by a variety of methods known to those of skill in the art. For example, the biological activity can be performed by measuring the relative infectivity in tissue culture with HT080 cells. In this procedure, blue colony forming unit (BCFU) titers are determined by counting wells for blue colonies after x-gal staining at two days post transduction. Titers can then be related to those of the unmodified vectors. (Current Protocols in Molecular Biology, Ausubel et al. eds.) Modification conditions are typically carefully determined on a trial basis in order to generate modified vector preparations with optimal retention of biological activity (at least 10-30% of the original activity).

Gene Delivery vehicles including retroviral vectors can be conjugated to large variety of polymers including dextran derivatives. Dextrans of low molecular weight are preferred, particularly dextrans of MW 1,000-10,000.

For example, polyaldehyde dextran can be prepared by NaIO4 oxidation according to the method of Bobbitt (*Adv. Carbohydrate Chem.* 11:1-43, 1956). This method results in the oxidization of the glucose rings with the generation of multiple aldehyde groups. Typically in this method, periodate oxidation proceeds for about 24 hours in the dark at room temperature. Oxidized dextrans can then dialyzed extensively and lyophilized. Polyaldehyde-dextrans can then be reacted with primary amines, such as the epsilon amino group of lysines of surface proteins of beta-gal vectors, to form Shiff bases, which can then stabilized by $NaCNBH_3$ reduction. Residual aldehyde groups on protein-bound dextrans can be reduced with $NaBH_4$. The level of dextran substitution of beta-gal vectors can be measured by a variety of techniques such as an anthrone colorimetric assay, based on the determination of reducing sugars (see Viles, F. J. Jr and Silverman, L., *Anal. Chem.* 21:950, 1949). This assay can also be used to determine the level of polyaldehyde content of periodate activated dextran.

Other derivatization conditions can be also be used, including the linkage of chain-terminal hydrazide-dextrans to the carbohydrate residues of surface proteins of vectors.

Gene delivery vehicles as used within the present invention refers to recombinant vehicles which contain nucleic acids that direct the expression on one or more heterologous nucleotide sequences, such as viral vectors (Jolly, *Cancer Gen. Therapy 1*:51-64, 1994). Representative examples of such gene delivery vehicles include poliovirus (Evans et al., *Nature 339*:385-388, 1989; and Sabin, *J. Biol. Standardization 1*:115-118, 1973); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); retrovirus (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242, and WO 91/02805); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *N. Eng. J. Med. 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); adenovirus (Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; WO 93/9191; Kolls et al., *PNAS 91*:215-219, 1994; Kass-Eisler et al., *PNAS 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; Guzman et al., *Cir. Res. 73*:1202-1207, 1993; Zabner et al., *Cell 75*:207-216, 1993; Li et al., *Hum. Gene. Ther. 4*:403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*:1287-1291, 1993; Vincent et al., *Nat. Genet. 5*:130-134, 1993; Jaffe et al., *Nat. Genet. 1*:372-378, 1992; and Levrero et al., *Gene 101*:195-202, 1991); parvovirus such as adeno-associated virus (Samulski et al., *J. Vir. 63*:3822-3828, 1989; Mendelson et al., *Virol. 166*:154-165, 1988; PA 7/222,684); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989); SV40; HIV (Poznansky, *J. Virol. 65*:532-536, 1991); measles (EP 0 440,219); astrovirus (Munroe, S.S. et al., *J. Vir. 67*:3611-3614, 1993); Semliki Forest Virus, and coronavirus, as well as other viral systems (*e.g.*, EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic (defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910,1988).

Within certain preferred embodiments, the gene delivery vehicle is a retrovirus. Retroviruses are RNA viruses with a single positive strand genome which, in general, are nonlytic. Upon infection, the retrovirus reverse transcribes its RNA genome into DNA, which is inserted into the host cell genome. Preparation of retroviral constructs for use in the present invention is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603, filed September 21, 1990), herein incorporated by reference. The retroviral genome can be divided conceptually into two parts. The "trans-acting" portion consists of the region coding for viral structural proteins, including the group specific antigen (*gag*) gene for synthesis of the core coat proteins; the *pol* gene for the synthesis of the reverse transcriptase and integrase enzymes; and the envelope (*env*) gene for the synthesis of envelope glycoproteins. The "cis-acting" portion

consists of regions of the genome that is finally packaged into the viral particle. These regions include the packaging signal, long terminal repeats (LTR) with promoters and polyadenylation signal sequences, and two start sites for DNA replication. The internal or "trans-acting" part of the cloned provirus is replaced by the gene of interest to create a "vector construct". When the vector construct is placed into a cell where viral packaging proteins are present (*see*, U.S.S.N. 07/800,921), the transcribed RNA will be packaged as a viral particle which, in turn, will bud off from the cell. These particles are used to transduce tissue cells, allowing the vector construct to integrate into the cell genome. Although the vector construct expresses its gene product, the virus carrying it is replication defective because the trans-acting portion of the viral genome is absent. Various assays may be utilized in order to detect the presence of any replication competent infectious retrovirus. One preferred assay is the extended S^+L^- assay.

Numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those disclosed within EP 415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram et al., *Cancer Res. 53*:83-88, 1993; Takamiya et al., *J. Neurosci. Res. 33*:493-503, 1992; Baba et al., *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 345,242 and WO 91/02805). Preferred retroviral vectors include murine leukemia amphotropic or xenotropic, or VSV-G pseudotype vectors (*see*, WO 92/14829, incorporated herein by reference).

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses including, for example, B-, C-, and D-type retroviruses, as well as spumaviruses and lentiviruses (*see*, RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2, and NZB_{9-1} (*see*, O'Neill et al., *J. Vir. 53*:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see*, Kelly et al., *J. Vir. 45*(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred retrovector constructs which are provided herein also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Within one aspect of the invention, retrovector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retrovector construct.

As an illustration, within one embodiment of the invention construction of retrovector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence beginning from the end of the 5' LTR up through the *Pst* I site. The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1560. Thus, within this embodiment, retrovector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal prior to nucleotide 567.

Within other embodiments of the invention, retrovector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated prior to the start of the *gag/pol* gene. Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615, or 617.

Within other aspects of the invention, retrovector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene. When

such retrovector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*.

Within other aspects of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the retrovector construct. Within a preferred embodiment, the retrovector constructs do not contain a *env* coding sequence upstream of the 5' LTR.

Within a further aspect of the present invention, retrovector constructs are provided, comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome.

Within another preferred embodiment, the gene delivery vehicle is a Sindbis viral vector. Briefly, the Sindbis virus is the prototype member of the alphavirus genus of the togavirus family. The unsegmented genomic RNA (49S RNA) of Sindbis virus is approximately 11,703 nucleotides in length, contains a 5' cap and a 3' polyadenylated tail, and displays positive polarity. Infectious enveloped Sindbis virus is produced by assembly of the viral nucleocapsid proteins onto the viral genomic RNA in the cytoplasm and budding through the cell membrane embedded with viral encoded glycoproteins. Entry of virus into cells is by endocytosis through clatharin coated pits, fusion of the viral membrane with the endosome, release of the nucleocapsid, and uncoating of the viral genome. During viral replication the genomic 49S RNA serves as template for synthesis of the complementary negative strand. This negative strand in turn serves as template for genomic RNA and an internally initiated 26S subgenomic RNA. The Sindbis viral nonstructural proteins are translated from the genomic RNA while structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as a polyprotein and processed into individual proteins by post-translational proteolytic cleavage. The

packaging sequence resides within the nonstructural coding region, therefore only the genomic 49S RNA is packaged into virions.

Several different Sindbis vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those described within U.S. Patent Nos. 5,091,309 and 5,217,879.

Particularly preferred Sindbis vectors for use within the present invention include those which are described within U.S. Serial No. 08/198,450. Briefly, within one embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and a Sindbis RNA polymerase recognition sequence. Within other embodiments, the viral junction region has been modified such that viral transcription of the subgenomic fragment is reduced. Within another embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which has been modified such that viral transcription of the subgenomic fragment is reduced, and a Sindbis RNA polymerase recognition sequence. Within yet another embodiment, Sindbis cDNA vector constructs are provided comprising the above-described vector constructs, in addition to a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination.

In still further embodiments, the vector constructs described above contain no Sindbis structural proteins in the vector constructs the selected heterologous sequence may be located downstream from the viral junction region; in the vector constructs described above having a second viral junction, the selected heterologous sequence may be located downstream from the second viral junction region, where the heterologous sequence is located downstream, the vector construct may comprise a polylinker located between the viral junction region and said heterologous sequence, and preferably the polylinker does not contain a wild-type Sindbis virus restriction endonuclease recognition sequence.

When Sindbis gene delivery vehicles are utilized, the modified cells are fully viable and healthy, and no other viral antigens (which may well be immunodominant) are expressed. The above-described Sindbis vector constructs, as well as numerous similar vector constructs, may be readily prepared essentially as described in U.S. Serial No. 08/198,450, which is incorporated herein by reference in its entirety.

As will be evident to one of ordinary skill in the art given the disclosure provided herein, when utilizing viral gene delivery vehicles, the efficiency of packaging and hence, viral titer, is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production particle virus, additional non-coding sequences may be added to the gene delivery vehicle. Moreover, within certain embodiments of the invention it may be desired to increase or decrease viral titer. This increase or decrease may be accomplished by increasing or decreasing the size of the heterologous sequence, and hence the efficiency of packaging.

Within one embodiment, gene delivery vehicles may be constructed to include a promoter such as SV40 (*see*, Kriegler et al., *Cell 38*:483, 1984), cytomegalovirus ("CMV") (*see*, Boshart et al., *Cell 41*:521-530, 1991), or an internal ribosomal binding site ("IRBS"). Briefly, with respect to IRBS, the upstream untranslated region of the immunoglobulin heavy chain binding protein has been shown to support the internal engagement of a bicistronic message (*see*, Jacejak and Sarnow, *Nature 353*:90-94, 1991). This sequence is small (300 bp), and may readily be incorporated into a retroviral vector or other gene delivery vehicle in order to express multiple genes from a multi-cistronic message whose cistrons begin with this sequence.

2. Identification of novel condensing agents

This invention also provides new condensing agents, with low or negligible immunogenicity, which are useful as condensing agents for nucleic acid. In one embodiment, the condensing agent is a basic amino acid region of at least 50 amino acids that has a predicted isoelectric point of at least 9, contains at least 40% basic amino acids, but not greater than 5% acidic amino acids, and preferably no acidic amino acids. Computer programs, such as Geneworks, version 2.4 (Intelligenetics), may be used as an aid in the calculation of isoelectric point.

A basic amino acid region is identified by its physical characteristics, predicted isoelectric point and percentage of basic amino acids. These regions may be derived from native proteins or contain a synthesized sequence not corresponding to a native protein. These regions must be at least 50 amino acids long, preferably longer than 90 residues, and particularly preferred longer than 200 amino acids. The total percentage of basic residues (generally, arginine plus lysine) for a native protein must be at least 40% and preferably greater than 50%. Histones and protamines are examples of proteins which have regions that satisfy these criteria. Once a basic region is identified, either the region may be synthesized based on the amino acid sequence, or the corresponding region of the gene may be isolated by conventional techniques, such as PCR amplification or restriction

enzyme digestion, before cloning and expression in bacteria, yeast, or other eukaryotic cells.

Proteins satisfying the criteria discussed above for basicity may be found by interrogating a database of protein sequences, such as PIR (Protein Identification Resource) or Swiss Prot. Database sequences are first scanned for proteins with a predicted isoelectric point of at least 8. Among those, the proteins are scanned for stretches of at least 50 amino acids containing at least 40% basic (generally, lysine plus arginine) residues, not greater than 5% acidic residues, and a predicted isoelectric point of at least 9. Alternatively, the database may be scanned in 50 amino acid blocks for stretches with the desired characteristics. A preferred length for a basic amino acid region is at least 90 amino acids and a particularly preferred length is at least 200 amino acids. Analysis indicates that proteins such as human serum albumin, DNA binding proteins, non-histone chromosomal proteins, proteins with nucleic acid binding capacity, or others, have regions exhibiting the requisite characteristics. Preferred regions are from the same species as the ultimate host for gene delivery.

Once such proteins are identified, the whole protein or basic domain, either in its native form or conjugated as described above, is tested for its ability to condense DNA, *in vitro* gene transfer efficiency, and for immunogenicity as complexed with nucleic acid.

A basic amino acid region is tested for *in vitro* gene transfer efficiency at a ratio that causes condensation of DNA as assayed in a band shift assay. If a single unit of basic amino acid region condensed DNA has at least 10% the efficiency of polylysine condensed DNA, it is tested in an immunogenicity assay. Preferably, *in vitro* gene transfer efficiency is at least 50% of the efficiency of polylysine.

Also within the scope of this invention, coat proteins from DNA viruses may serve as nucleic acid condensing agents. Coat proteins bind DNA and assist in the packaging of viral genomes into viral capsids. One coat protein, from ϕX174, is known to condense DNA. Other coat proteins may be prepared chemically or biologically and tested for their ability to condense DNA, transfer genes *in vitro*, and for immunogenicity.

Transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic acid condensing agents. Briefly, transcriptional factors such as C/CEBP, c-*jun*, c-*fos*, AP-1, AP-2, AP-3, CPF, Prt-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences. As discussed above, the basic domain may be prepared by PCR amplification, isolated by restriction enzyme digestion, or chemically synthesized. Single units are tested for nucleic acid condensation, *in vitro* gene transfer efficiency, and immunogenicity.

If single units of basic amino acid regions derived from identified basic proteins, such as chromosomal proteins, transcription factors, DNA virus coat proteins, or other proteins are less than 10% as efficient as polylysine in gene transfer assays, multiple units may be synthesized and tested for sufficient gene transfer efficiency.

One embodiment of this invention provides a condensing agent that contains multiple units of basic domains from native protein. Briefly, a region from a basic protein is identified as described above. DNA molecules encoding the basic region may be ligated together in a head-to-tail or head-to-head fashion following the addition of adapters containing appropriate restriction sites. From 1-10 units are composed in an array, preferably 1-5 units, most preferably 1-2. Alternatively, restriction sites may be added during amplification of the region by incorporating a restriction site sequence into the primers used in amplification. Following ligation of the units, molecules containing the desired number of repeat units may be isolated following gel electrophoresis. These units are then inserted into an appropriate expression vector (which may be commercially obtained), propagated, and protein isolated by conventional techniques. Alternatively, multiple units may be generated chemically by synthesizing single units of basic amino acid regions and chemically or enzymatically coupling them together.

ANALYSIS OF CONDENSING AGENTS

Once condensing agents have been prepared as described above, they are tested for several different parameters. First, the candidate condensing agent must be able to condense nucleic acids. Second, nucleic acids are condensed by the agent and tested in an *in vitro* gene transfer assay. Third, the agent is tested as part of a condensed nucleic acid-complex immunogenicity assay, both for induction of antibodies and inactivation by a serum component. Only condensing agents satisfying the criteria of the first, second, and either part of the third assay are selected as condensing agents with low immunogenicity. Thus, the nucleic acid condensing agents identified as described herein possess low or negligible immunogenicity, while retaining substantial efficiency of gene transfer activity.

1. Condensation

Agents which have been modified or newly identified are assayed for their ability to condense nucleic acid utilizing at least two different assays. An intercalating dye assay may be used as an optional, preliminary screen. All candidate condensing agents are assayed by a DNA band shift assay. Both assays are based upon changes in the physical properties of DNA when it is condensed. Condensation "collapses" DNA into macromolecular structures, commonly into a toroid form. In this condensed state, DNA

will not intercalate as much ethidium bromide or other intercalating dye and has reduced mobility in agarose gel electrophoresis.

As an illustrative example, an intercalating dye assay using ethidium bromide is described. In this assay, test nucleic acids, conveniently plasmid DNA, are mixed with candidate condensing agents from a 1:1 to a 1:50 w/w ratio of plasmid to condensing agent. Following incubation, ethidium bromide is added to the reaction to a final concentration of 1 μg/ml. If a nucleic acid such as RNA is used as the test nucleic acid, acridine orange may be used as the intercalating dye. The reaction mixtures are transferred into UV transparent plastic tubes spotted onto 1% agarose gel, or placed upon UV transparent plastic film and illuminated with 260 nm light. The emission from the DNA-ethidium bromide complex is recorded on film by a camera equipped with an appropriate UV filter. The ability of the candidate condensing agent to condense DNA is inversely proportional to the intensity of the fluorescence in each reaction mixture. A condensing agent which either reduces fluorescence intensity by at least 10-fold relative to uncondensed plasmid or reduces fluorescence intensity to 50% of the reduction caused by polylysine condensation is selected for further analysis.

The more precise test, and the one performed for each candidate condensing agent, is a band shift assay. Briefly, this assay is performed by incubating nucleic acids, either labeled or unlabeled, with various concentrations of candidate condensing agents. Test nucleic acids, conveniently plasmid DNA, and condensing agent are mixed at 1:1 to 1:50 w/w ratios. Following incubation, each sample is loaded on a 1% agarose gel and electrophoresed. The gel is then either stained with ethidium bromide or dried and autoradiographed. DNA condensation is determined by the inability to enter the gel compared to a non-condensed standard. Sufficient condensation is achieved when at least 90% of the DNA fails to enter the gel to any significant degree.

2. Efficiency of Gene Transfer

A variety of *in vitro* methods may be utilized in order to assess the efficacy of gene transfer with these nucleic acid condensing agents. For example, an *in vitro* test may be performed using a plasmid construct containing a reporter gene such as β-gal, luciferase, CAT, or human growth hormone. An appropriate recipient cell line is chosen for the assay. This cell line is preferably a cell line with properties similar to the cells to which the gene will be targeted in patients. However, the recipient cells should not express significant levels of the same gene product that is contained on the test plasmid. Of the various reporter genes, luciferase is used herein as an example. Briefly, the reporter gene construct is mixed with condensing agents. Following an incubation period, the mixtures are added to the recipient cells. Cells are harvested 1 day after transfer. The levels of

luciferase are determined using an assay kit (Promega Corp. Part No. E1500) according to manufacturer's instructions.

Gene transfer efficiency of the plasmid condensed with the candidate condensing agent is compared to the efficiency obtained with the plasmid condensed with polylysine having an average length of 270 amino acids. Agents that mediate gene transfer with an efficiency of at least 10%, and preferably 50% or more, relative to polylysine are chosen for further analysis.

3. Immunogenicity

Following the identification of agents which condense DNA and determination of the efficiency of gene transfer *in vitro*, these agents are assayed for immunogenicity. *In vivo* tests may be performed and are generally preferred. In assessing whether an agent elicits an immune response, the most important responses to evaluate are an antibody response and other humoral factors which may interfere with *in vivo* function, either initially or after repeated doses to a subject.

Nucleic acid condensed with the modified condensing agent or novel condensing agent is introduced *in vivo* and an antibody immune response determined. If the total antibody response as measured by ELISA is sufficiently low, the candidate agent is selected. If this antibody response is not sufficiently low, sera is tested in a neutralization assay. If the antibodies are neutralizing, the candidate is discarded. If the antibodies are not neutralizing, the candidate agent is selected. Selected candidates may be tested further for inactivation by a serum component.

The antibody immune response is tested differently for condensing agents that are conjugated polycations and for condensing agents that are basic amino acid regions. For the first type of condensing agents, modifications of a particular condensing agent/nucleic acid complex relative to the unmodified complex may be initially and optionally tested by an *in vitro* test, such as an ELISA as described below, in order to screen for the most promising modifications of condensing agents to be tested by *in vivo* assays. For all types of agents, *in vivo* determinations of immunogenicity are performed and preferred. In assessing the ability of an agent to elicit an immune response, an antibody response is a critical parameter to evaluate as described in Example 5. Additionally, other parameters to be evaluated include assessment of other humoral factors, such as complement. An example of an assay to address the presence of these factors is set forth in Example 6. These factors may interfere with *in vivo* function either initially or after repeated administrations of a particular condensing agent/nucleic acid complex.

In an *in vitro* assay, antibody reactivity to a specific conjugated nucleic acid condensing agent/nucleic acid complex is compared to the unmodified condensing agent

complex in a standard ELISA. Wells are coated with a polyclonal antibody specific for the unconjugated condensing agent/nucleic acid complex (*i.e.*, antibody obtained from sera of rabbits multiply injected with polylysine/nucleic acid with or without an adjuvant). Injections are continued until an adequate antibody titer is obtained in serum. Alternatively, but less preferably, a monoclonal antibody may be used. Conjugations which decrease the binding by at least three fold, or ten-fold preferably, relative to the binding to unmodified condensing agent/nucleic acid complex will be chosen as promising candidates for *in vivo* testing.

An *in vivo* assay for immunogenicity is performed by introducing the conjugated condensing agent or novel condensing agent *in vivo* and determining an antibody immune response. In this assay, mice, rabbits, or preferably macaques or other primates, are repeatedly injected with nucleic acids condensed with one of the condensing agents described above. As an illustration, a plasmid, such as pGL2, is condensed with PEG-conjugated polylysine and injected i.m., i.v., i.p., or s.c. into a group of three or more young adult mice. A total of three injections are given with an interval between injections ranging from two weeks to two months. The dose per injection in each group may be based on projection of the approximate human dose equivalent for the specific gene and gene transfer application being considered and may be bracketed 3-10 fold higher and lower in parallel groups. Alternatively, the dose may be chosen arbitrarily at 100 mg/kg and may be bracketed 3-10 fold higher and lower for alternative groups, or the dose may be established by first determining a dose of unconjugated polycation that gives a measurable antibody response. Serum is isolated from blood samples collected at 1, 2, 3, 4, and 8 weeks after the final injection. Antibody titers against the condensing agent are measured by ELISA and compared to antibody titers against unconjugated polylysine-condensed complexes obtained from mice injected with these complexes. Lowered immunogenicity is defined as a reduction of induced antibody titer of at least three-fold, and preferably ten-fold or greater relative to the polylysine/nucleic acid complex injected animal, at matched DNA dosage. Satisfying this criteria at any of the dose/interval combinations tested in an animal system is sufficient to show low immunogenicity within the context of this invention.

In the case of the novel condensing agents, immunogenicity studies are preferably done in animals closely related to, and most preferably identical, to the species for which the gene transfer complex is intended. That is, gene transfer complexes using novel condensing agents which are intended for human use are tested for *in vivo* immunogenicity preferably in primates and most preferably in humans. In such cases, the control standard against which immunogenicity comparisons are based remains the nucleic acid/unconjugated-polylysine complex.

Because measurements of total antibody by ELISA do not measure the level of antibody that is "neutralizing" (i.e., prevents the complex from achieving gene transfer) selection of condensing agents that are immunogenic may be justified if antibodies do not inhibit gene transfer. Interference with *in vitro* gene transfer efficiency is determined after treatment with heat-inactivated sera from multiply-injected animals and after treatment with heat-inactivated FBS. Efficiencies are determined for DNA complexed with the candidate condensing agent as well as with polylysine. The relative efficiency is calculated as a percentage for each condensing agent in the presence of immune sera compared to FBS. A nucleic acid/condensing agent complex will meet the criteria for low immunogenicity if its relative efficiency is at least three-fold, and preferably ten-fold, higher than the relative efficiency for the nucleic acid/polylysine complex.

If the condensing agent does not have low immunogenicity by ELISA after *in vivo* administration or elicits neutralizing antibodies, it may still be considered useful within the context of this invention, if it is not inactivated by a serum component. Such an agent may be used when a single dose of gene therapy is to be administered. Inactivation by a serum component may be mediated by one of the proteins of the complement system. Briefly, in this assay, nucleic acid, conveniently a plasmid expressing β-galactosidase, such as pSV-β Galactosidase (obtainable from Promega, Cat# E1081), is complexed with the candidate nucleic acid condensing agent. The complex is then treated with heat-inactivated fetal bovine sera (as a control), human sera, or heat-inactivated human sera. Heat-inactivated sera are prepared by heating to 56°C for 30 minutes. These mixtures are then incubated at 37°C for 30 minutes. Transfection efficiency is determined by a standard blue colony forming unit (BCFU) assay (*Current Protocols in Molecular Biology, supra*). Reduced sensitivity to inactivation and thus, low immunogenicity, is defined as an increase of two-fold or better survival relative to polylysine-condensed DNA. Survival is expressed as BCFU/ml after treatment with heat-inactivated serum relative to treatment with FBS.

The immunogenicity of polymer-modified vectors can be evaluated in a variety of animal models by standard immunology techniques. (See Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Publications (1988.) Typically, an immunization and bleeding schedule is first determined. Prior to the initiation of the experiment, animals can be pre-bled and their preexisting anti-vector titres determined , for instance, with an ELISA assay.

Animals can be injected with the modified vectors of the invention and the immune responses can be determined by immunological methods. For example, the immunogenicity of modified retroviral vectors in mice can be determined by measuring the extent of the anti-vector immune response in mouse sera with an ELISA assay (Engvall, E. *Meth. Enzymol* 70:419, 1980). In this particular assay, microtiter well plates are coated

with the retroviral vector. Non-specific binding sites are blocked with bovine serum albumin (BSA), and wells are exposed to serial dilutions of either individual or pooled mouse sera. The wells are washed thoroughly and incubated with commercially available goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP). The color is developed with the ortho-phenyldiamine in hydrogen peroxide solution.

A variety of other immunoassay methods can also be used. (See eg. Enzyme Immunoassay, E.T. Maggio, editor, CRC Press, Baca Raton, Florida (1980.) For instance, a capture ELISA assay can be set up in the following manner. Microtiter well plates can be coated with serial dilutions of mouse sera, and non specific binding sites can be blocked with a solution of BSA. A preparation of retroviral vector is then added to the wells, which are washed thoroughly and exposed to monoclonal anti-vector antibodies. The wells are then treated with commercially available goat anti-mouse antibodies conjugated with either HRP or AP, and the assay is performed as described above.

Synthetic polymers are known to exhibit varying levels of immunogenicity depending upon the polymer itself, the type of conjugation to the protein, or the nature of the protein carrier. Dextrans of high molecular weight (>95,000) are known to be immunogenic, especially when bound to protein carriers (Kabat, E. A. and Berg, D.J. *Immunology* 70:514, 1953; Richter, W. and Kagedal, L. *Int. Arch. Allergy* 42:885, 1972). Therefore, the immune response against the dextran portion of dextran-modified vectors can also evaluated in sera of animals with a modification of the immunuization procedures and immunoassays described above. For example, microtiter well plates can be coated with unmodified vector, dextran-modified vector, BSA, and dextran-modified BSA. Following a wash, non-specific binding sites are blocked with a BSA solution, and the wells are exposed to serum samples, either individually or pooled. The wells are again washed thoroughly. A preparation of rabbit anti-BSA IgG can be used as a control and the immunogenicity of the synthetic polymer can be calculated.

ADDITIONAL COMPONENTS IN NUCLEIC ACID/CONDENSING AGENT COMPLEXES

Within various aspects of the invention, a wide variety of ligands are provided. Ligands which bind to a cell surface protein, may be included in the complex to facilitate targeting of the condensed nucleic acid complex to a particular cell type. Such ligands include, for example, transferrin, asialoglycoprotein, antibody and antibody fragments, bombesin, gastrin-release peptide, cell adhesion peptides, substance P, neuromedin-B, neuromedin-C, metenkephalin, EGF, alpha- and beta-TGF, neurotensin, melanocyte stimulating hormone, follicle stimulating hormone, luteinizing hormone, human

growth hormone, cell surface receptors, low density lipoproteins, transferrin, erythropoietin, insulin and fibrinolytic enzymes. Other targeting elements include immune accessory molecules, which include, for example, the interleukins IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, α-interferon, β-interferon, γ-interferon, GM-CSF, G - CSF, G-CSF and M-CSF. With the aforementioned ligands, a desired cell type which expresses the binding partner is targeted due to the choice of the appropriate ligand. Alternatively, one member of a wide variety of high affinity binding pairs may also be used as a ligand. These include, for example, biotin/avidin, cytostatin/papain, valphosphonate/carboxypeptidase A, 4CABP/RuBisCo, and tobacco hornworm diuretic hormone/tobacco hornworm diuretic hormone receptor, as well as antigen/antibody binding pairs. When using these high affinity binding pairs one member of the pair is incorporated into the nucleic acid/condensing agent complex and the other member attached to the cell of interest. The ligands may be coupled to the condensing agent by methods described in U.S. Patent Nos. 5,166,320 and 5,354,844 (incorporated herein by reference).

Additionally, components which disrupt endosomes may be included in the complex. For example, the capsid proteins of adenoviruses cause acidification of an endosome and ultimate disruption, thereby releasing the introduced nucleic acid (Defer et al., *J. Virol. 64*:3661, 1990; Seth et al., *Mol. Cell. Biol. 4*:1528, 1984; Fitzgerald et al., *Cell 32*:607, 1983). Through this mechanism, adenovirus can greatly increase the efficiency of gene transfer (Curiel et al., *Proc. Natl. Acad. Sci USA 88*:8850, 1991). Other endosomolytic agents may alternatively be incorporated into the complex to achieve an endosome breakdown. Other agents, such as lysomotropic agents (e.g., chloroquine), may be included to decrease DNA degradation that occurs in lysosomes.

NUCLEIC ACIDS

A nucleic acid molecule administered to an animal in accordance with the present invention does not naturally occur in the complex or gene delivery vehicle that carries it, and is neither inert nor generally harmful to the animal, but rather provides some desirable benefit, typically an ability to fight a disease or other pathogenic agent. As used herein, "pathogenic agent" refers to a cell that is responsible for a disease state. Representative examples of pathogenic agents include tumor cells, autoreactive immune cells, hormone secreting cells, cells which lack a function that they would normally have, cells that have an additional inappropriate gene expression which does not normally occur in that cell type, and cells infected with bacteria, viruses, or other intracellular parasites. In addition, as used herein, "pathogenic agent" may also refer to a cell that over-expresses or

inappropriately expresses a retroviral vector (*e.g.*, in the wrong cell type), or that has become tumorigenic due to inappropriate insertion into a host cell's genome.

A wide variety of nucleic acid molecules may be carried by the complex or gene delivery vehicle of the present invention. Examples of such nucleic acid molecules include genes and other nucleic acid molecules that encode a substance, as well as biologically active nucleic acid molecules such as inactivating sequences that incorporate into a specified intracellular nucleic acid molecule and inactivate that molecule. A nucleic acid molecule is biologically active when the molecule itself provides the desired benefit without requiring the expression of a substance. For example, the biologically active nucleic acid molecule may be an inactivating sequence that incorporates into a specified intracellular nucleic acid molecule and inactivates that molecule, or the molecule may be a tRNA, rRNA or mRNA that has a configuration that provides a binding capability.

Substances include proteins (*e.g.*, antibodies including single chain molecules), immunostimulatory molecules (such as antigens) immunosuppressive molecules, blocking agents, and palliatives (such as toxins, antisense ribonucleic acids, ribozymes, enzymes, and other material capable of inhibiting a function of a pathogenic agent). Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (*e.g.*, contact inhibition), and lack of production of anti-oncogene proteins.

Within one embodiment of the present invention, a method is provided for administration of various nucleic acids, such as eukaryotic viral cDNA expression vectors, which direct the expression of a palliative as a DNA molecule. Within another embodiment of the present invention, a method is provided for administration of various nucleic acids which direct the expression of a palliative as an RNA molecule.

Representative examples of palliatives that act directly to inhibit the growth of cells include toxins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen et al., *J. of Biol. Chem. 266*:6848-6852, 1991; Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed toxin (Irvin, *Pharmac. Ther. 21*:371-387, 1983),

antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and *Pseudomonas* exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within other aspects of the invention, the nucleic acid carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant retrovirus nucleic acid could carry a gene encoding a proprotein chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

Within yet another aspect of the invention, the nucleic acid directs the expression of a substance capable of activating an otherwise inactive precursor into an active inhibitor of a pathogenic agent, or a conditional toxic palliative, which are palliatives that are toxic for the cell expressing the pathogenic condition. As will be evident to one of skill in the art given the disclosure provided herein, a wide variety of inactive precursors may be converted into active inhibitors of a pathogenic agent. For example, antiviral nucleoside analogues such as AZT or ddI are metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus viral replication (Furmam et al., *Proc. Natl. Acad. Sci. USA 83*:8333-8337, 1986). Recombinant viral vectors which direct the expression of a gene product (*e.g.*, a protein) such as Herpes Simplex Virus Thymidine Kinase (HSVTK) or Varicella Zoster Virus Thymidine Kinase (VZVTK) which assists in metabolizing antiviral nucleoside analogues to their active form are therefore useful in activating nucleoside analogue precursors (*e.g.*, AZT or ddC) into their active form. AZT or ddI therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Within one embodiment of the invention, the HSVTK gene may be expressed under the control of a constitutive macrophage or T-cell-specific promoter, and introduced into macrophage or T-cells. Constitutive expression of HSVTK results in more effective metabolism of nucleotide analogues such as AZT or ddI to their biologically active nucleotide triphosphate form, and thereby provides greater efficacy, delivery of lower

doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, may also be utilized within the context of the present invention.

Within a related aspect of the present invention, a nucleic acid directs the expression of a substance that activates another compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localized therapy to the pathogenic agent. In this case, expression of the gene product from the nucleic acid is limited to situations wherein an entity associated with the pathogenic agent, such as an intracellular signal identifying the pathogenic state, is present, thereby avoiding destruction of nonpathogenic cells. This cell-type specificity may also be conferred at the level of infection, by targeting the nucleic acid carrying the vector to cells having or being susceptible to the pathogenic condition.

Within a related aspect of the present invention, a nucleic acid directs the expression of a gene product(s) that activates a compound with little or no cytotoxicity into a toxic product. Briefly, a wide variety of gene products which either directly or indirectly activate a compound with little or no cytotoxicity into a toxic product may be utilized within the context of the present invention. Representative examples of such gene products include HSVTK and VZVTK which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs gancyclovir, acyclovir, or any of their analogues (*e.g.*, FIAC, DHPG) to HSVTK, phosphorylates the drug into its corresponding active nucleotide triphosphate form.

For example, within one embodiment of the invention, the nucleic acid directs the expression of the herpes simplex virus thymidine kinase ("HSVTK") gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the nucleic acid causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as gancyclovir, acyclovir or its analogues (FIAC, DHPG). As noted above, these drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., *Proc. Natl. Acad. Sci. USA 85*:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within one embodiment of the invention, expression of a conditionally lethal HSVTK gene may be made even more HIV-specific by including cis-acting elements in the transcript ("CRS/CAR"), which require an additional HIV gene product, rev, for optimal activity (Rosen et al., *Proc. Natl. Acad. Sci. USA 85*:2071, 1988). More generally, cis elements present in mRNAs have been shown in some cases to regulate mRNA stability or translatability. Sequences of this type (*i.e.*, post-transcriptional regulation of gene expression) may be used for event- or tissue-specific regulation of vector gene expression. In addition, multimerization of these sequences (*i.e.*, rev-responsive "CRS/CAR" or tat-responsive "TAR" elements for HIV) may be utilized in order to generate even greater specificity.

In a manner similar to the preceding embodiment, nucleic acids may be generated which carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. Such genes may have no equivalent in mammalian cells, and might come from an organism such as a virus, bacterium, fungus, or protozoan. Representative examples include: *E. coli* guanine phosphoribosyl transferase ("gpt") gene product, which converts thioxanthine into thioxanthine monophosphate (see Besnard et al., *Mol. Cell. Biol. 7*:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (*e.g.*, *Fusarium oxysporum*) or bacterial cytosine deaminase which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS 89*:33, 1992); carboxypeptidase G2 which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxyacetabide derivatives of doxorubicin and melphalan to toxic compounds. Conditionally lethal gene products of this type have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., *Brit. J. Cancer 53*:377-384, 1986).

Additionally, in the instance where the target pathogen is a mammalian virus, the nucleic acid may be constructed to take advantage of the fact that mammalian viruses in general tend to have "immediate early" genes, which are necessary for subsequent transcriptional activation of other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes transcribed from transcriptional promoter elements that are responsive to such viral "immediate early" gene products could specifically kill cells

infected with any particular virus. Additionally, since the human α and β interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VREs) could result in the destruction of cells infected with a variety of different viruses.

In another embodiment of the invention, methods are provided for producing substances such as inhibitor palliatives involving the delivery and expression of defective interfering viral structural proteins, which inhibit viral assembly. In this context, nucleic acid codes for defective gag, pol, env or other viral particle proteins or peptides which inhibit in a dominant fashion the assembly of viral particles. Such inhibition occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

One way of increasing the effectiveness of inhibitory palliatives is to express inhibitory genes, such as viral inhibitory genes, in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, a nucleic acid may be administered that inhibits HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

In another embodiment of the invention, methods are provided for the expression substances such as inhibiting peptides or proteins specific for viral protease. Viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. Nucleic acids that inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

The approaches discussed above should be effective against many virally linked diseases, cancers, or other pathogenic agents.

Within still other embodiments of the invention, a nucleic acid is provided that expresses a palliative, wherein the palliative has a membrane anchor and acts as an anti-tumor agent(s). Such a palliative may be constructed, for example, as an anti-tumor agent - membrane anchor fusion protein. Briefly, the membrane anchor aspect of the fusion protein may be selected from a variety of sequences, including, for example, the transmembrane domain of well known molecules. Generally, membrane anchor sequences

are regions of a protein that bind the protein to a membrane. Customarily, there are two types of anchor sequences that attach a protein to the outer surface of a cell membrane: (1) transmembrane regions that span the lipid bilayer of the cell membrane, and interact with the hydrophobic center region (proteins containing such regions are referred to integral membrane proteins), and (2) domains which interact with an integral membrane protein or with the polar surface of the membrane (such proteins are referred to as peripheral, or extrinsic, proteins).

Membrane anchors for use within the present invention may contain transmembrane domains which span the membrane one or more times. For example, in glycophorin and guanylyl cyclase, the membrane binding region spans the membrane once, whereas the transmembrane domain of rhodopsin spans the membrane seven times, and that of the photosynthetic reaction center of *Rhodopseudomonas viridis* spans the membrane eleven times (*see* Ross et al., *J. Biol. Chem. 257*:4152, 1982; Garbers, *Pharmac. Ther. 50*:337-345, 1991; Engelman et al., *Proc. Natl. Acad. Sci. USA 77*:2023, 1980; Heijne and Manoil, *Prot. Eng. 4*:109-112, 1990). Particularly preferred membrane anchors for use within the present invention include naturally occurring cellular proteins (that are non-immunogenic) which have been demonstrated to function as membrane signal anchors (such as glycophorin).

Within a preferred embodiment of the present invention, a DNA sequence is provided which encodes a membrane anchor - gamma interferon fusion protein. Within one embodiment, this fusion protein may be constructed by genetically fusing the sequence which encodes the membrane anchor of the gamma-chain of the Fc receptor, to a sequence which encodes gamma-interferon.

In yet another aspect, the nucleic acid provides a therapeutic effect by encoding one or more ribozymes (RNA enzymes) (Haseloff and Gerlach, *Nature 334*:585, 1989) which will cleave, and hence inactivate, RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA sequence corresponding to a pathogenic state, such as HIV tat, and toxicity is specific to such pathogenic state. Additional specificity may be achieved in some cases by making this a conditional toxic palliative, as discussed above.

In still another aspect, the nucleic acid comprises a biologically active nucleic acid molecule that is an antisense sequence (an antisense sequence may also be encoded by a nucleic acid sequence and then produced within a host cell via transcription). In preferred embodiments, the antisense sequence is selected from the group consisting of sequences which encode influenza virus, HIV, HSV, HPV, CMV, and HBV. The antisense sequence may also be an antisense RNA complementary to RNA sequences necessary for

pathogenicity. Alternatively, the biologically active nucleic acid molecule may be a sense RNA (or DNA) complementary to RNA sequences necessary for pathogenicity.

More particularly, the biologically active nucleic acid molecule may be an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein, or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys. 253*:214-220, 1987; Bzik et al., *PNAS 84*:8360-8364, 1987), antisense HER2 (Coussens et al., *Science 230*:1132-1139, 1985), antisense abl (Fainstein et al., *Oncogene 4*:1477-1481, 1989), antisense myc (Stanton et al., *Nature 310*:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

In addition, within a further embodiment of the invention antisense RNA may be utilized as an anti-tumor agent in order to induce a potent class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

In another embodiment, the substances of the invention include a surface protein that is itself therapeutically beneficial. For example, in the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly

applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

Still further aspects of the present invention relate to the administration of a nucleic acid capable of immunostimulation. The ability to recognize and defend against foreign pathogens is essential to the function of the immune system. In particular, the immune system must be capable of distinguishing "self" from "nonself" (*i.e.*, foreign), so that the defensive mechanisms of the host are directed toward invading entities instead of against host tissues. Cytolytic T lymphocytes (CTLs) are typically induced, or stimulated, by the display of a cell surface recognition structure, such as a processed, pathogen-specific peptide, in conjunction with a MHC class I or class II cell surface protein.

Diseases suitable to treatment include viral infections such as HIV, HBV and HPV, cancers such as melanomas, renal carcinoma, breast cancer, ovarian cancer and other cancers, and heart disease.

In one embodiment, the invention provides methods for stimulating a specific immune response and inhibiting viral spread by using nucleic acids that direct the expression of an antigen or modified form thereof in susceptible target cells, wherein the antigen is capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant viral vector is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogs thereof (*e.g.*, Altmann et al., *Nature 338*:512, 1989). In accordance with a preferred embodiment, cells infected with Sindbis viral vectors are expected to do this efficiently because they closely mimic genuine viral infection and (a) are able to infect non-replicating cells; (b) do not integrate into the host cell genome; and (c) are not associated with any life threatening diseases.

This embodiment of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and no other viral antigens (which may well be immunodominant) are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant Sindbis virus, leading to responses against immunogenic

epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular generation and association of these peptide fragments with MHC class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response can also be achieved by transferring to an appropriate immune cell (such as a B or T lymphocyte) (a) the gene for the specific T-cell receptor that recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), (b) the gene for an immunoglobulin which recognizes the antigen of interest, or (c) the gene for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus the nucleic acid may be used as an immunostimulant, immunomodulator, or vaccine, etc.

In the particular case of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from the recombinant retroviral genome is of a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120, and gp 41, which have been modified to reduce their pathogenicity. In particular, the selected antigen is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but haplotype-specific epitopes or to present several haplotype-specific epitopes, and allow a response capable of eliminating cells infected with most or all strains of HIV. The haplotype-specific epitopes can be further selected to promote the stimulation of an immune response within an animal which is cross-reactive against other strains of HIV. Antigens from other HIV genes or combinations of genes, such as *gag, pol, rev, vif, nef, prot, gag/pol, gag prot,* etc., may also provide protection in particular cases.

HIV is only one example. This approach should be effective against many virally linked diseases or cancers where a characteristic antigen (which does not need to be a membrane protein) is expressed, such as in HPV and cervical carcinoma, HTLV-I-induced leukemias, prostate-specific antigen (PSA) and prostate cancer, mutated p53 and colon carcinoma and melanoma, melanoma specific antigens (MAGEs), and melanoma, mucin and breast cancer.

In accordance with the immunostimulation aspects of the invention, the substances of the present invention may also include "immunomodulatory factors," many of

which are set forth above. Immunomodulatory factors refer to factors that, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factors. The factors may also be expressed from a non-nucleic acid derived gene, but the expression is driven or controlled by the nucleic acid. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, ^{3}H thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure ^{51}Cr release) (*see*, Warner et al., *AIDS Res. and Human Retroviruses 7*:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*.

Representative examples of such factors include cytokines, such as IL-1, IL-2 (Karupiah et al., *J. Immunology 144*:290-298, 1990; Weber et al., *J. Exp. Med. 166*:1716-1733, 1987; Gansbacher et al., *J. Exp. Med. 172*:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-3, IL-4 (Tepper et al., *Cell 57*:503-512, 1989; Golumbek et al., *Science 254*:713-716, 1991; U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof et al., *J. Immunol. 139*:4116-4121, 1987; WO 90/06370), IL-7 (U.S. Patent No. 4,965,195) , IL-8, IL-9, IL-10, IL-11, IL-12, IL-13 (*Cytokine Bulliten*, Summer 1994), particularly IL-2, IL-4, IL-6, IL-12, and IL-13, alpha interferon (Finter et al., *Drugs 42*(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz. 78*:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA 86*:2046-2050, 1989; Faktor et al., *Oncogene 5*:867-872, 1990), beta interferon (Seif et al., *J. Virol. 65*:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 20082015, 1991; Watanabe et al., *PNAS 86*:9456-9460, 1989; Gansbacher et al., *Cancer Research 50*:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother. 30*:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman et al., *J. Immunology 144*:942-951, 1990), CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), ICAM-1 (Altman et al., *Nature 338*:512-514, 1989; Simmons et al., *Nature 331*:624-627, 1988), ICAM-2, LFA-1, LFA-3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), MHC class I molecules, MHC class II molecules, B7.1-.3, β_2-microglobulin (Parnes et al., *PNAS 78*:2253-2257, 1981), chaperone-like molecules, such as calnexin, MHC-linked transporter proteins or analogs thereof (Powis et al., *Nature 354*:528-531, 1991). Within one preferred embodiment, the gene encodes gamma-interferon.

An example of an immunomodulatory factor cited above is a member of the B7 family of molecules (*e.g.*, B7.1-.3 costimulatory factor). Briefly, activation of the full

functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen presenting cells. The second signal is required for interleukin-2 (IL-2) production by T cells, and appears to involve interaction of the B7.1-.3 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med.*, *173*:721-730, 1991a and *J. Exp. Med.*, *174*:561-570, 1991). Within one embodiment of the invention, B7.1-.3 may be introduced into tumor cells in order to cause costimulation of $CD8^+$ T cells, such that the $CD8^+$T cells produce enough IL-2 to expand and become fully activated. These $CD8^+$ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7.1-.3 factor, and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen presenting cells. The HBV core-specific CTL response will be augmented from the fully activated $CD8^+$ T cell via the costimulatory ligand B7.1-.3.

The choice of which immunomodulatory factor to include within a nucleic acid may be based upon known therapeutic effects of the factor, or, experimentally determined. For example, a known therapeutic effector in chronic hepatitis B infections is alpha interferon. This has been found to be efficacious in compensating a patient's immunological deficit, and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory factor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA matched cells (*e.g.*, EBV transformed cells) that have been transduced with a nucleic acid which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory factor. These stimulated PBLs are then used as effectors in a CTL assay with the HLA matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory factor. Within one embodiment of the invention, the immunomodulatory factor gamma interferon is particularly preferred.

The present invention also includes immunogenic portions of desired antigens. For example, various immunogenic portions of the HBV S antigens may be combined in order to present an immune response when administered by one of the nucleic acids described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S antigen open reading frame of HBV, particular

combinations of antigens may be preferred for administration in particular geographic regions.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected liver tissue. The polymerase protein consists of at least two domains: the amino terminal domain encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods utilizing nucleic acids administered in order to generate an immune response within an animal, preferably a warm-blooded animal. Similarly, other HBV antigens such as ORF 5 and ORF 6, (Miller et al., *Hepatology 9*:322-327, 1989), may be expressed utilizing nucleic acids as described herein.

As noted above, at least one immunogenic portion of a hepatitis B antigen can be incorporated into a nucleic acid. The immunogenic portion(s) which are incorporated into the nucleic acid may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long, and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). From this analysis, peptides may be synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

Within one embodiment of the present invention, at least one immunogenic portion of a hepatitis C antigen can be incorporated into a nucleic acid. Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology 14*:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polypeptide may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. *(Nature 351:290, 1991)*. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay.

Within another aspect of the present invention, methods are provided for destroying hepatitis B carcinoma cells comprising the step of administering to a warm-

blooded animal a nucleic acid which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated. Sequences which encode the HBxAg may readily be obtained by one of skill in the art given the disclosure provided herein. Briefly, within one embodiment of the present invention, a 642 bp *Nco* I-*Taq* I fragment is recovered from ATCC 45020, and inserted into nucleic acids as described above for other hepatitis B antigens.

Within another aspect of the present invention, methods are provided for destroying hepatitis C carcinoma cells comprising the step of administering to a warm-blooded animal a nucleic acid which directs the expression of an immunogenic portion of a hepatitis C antigen. Preferred immunogenic portion(s) of a hepatitis C antigen may be found in the polypeptide which contains the core antigen and the NS1-NS5 regions (Choo et al., *Proc. Natl. Acad. Sci. USA 88*:2451-2455, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above preferred immunogenic portions may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). Another method that may also be utilized to predict immunogenic portions is to determine which portion has the property of CTL induction in mice utilizing retroviral vectors (*see*, Warner et al., *AIDS Res. and Human Retroviruses 7*:645-655, 1991). As noted within Warner et al., CTL induction in mice may be utilized to predict cellular immunogenicity in humans. Preferred immunogenic portions may also be deduced by determining which fragments of the polypeptide antigen or peptides are capable of inducing lysis by autologous patient lymphocytes of target cells (*e.g.*, autologous EBV-transformed lymphocytes) expressing the fragments after vector transduction of the corresponding genes.

Preferred immunogenic portions may also be selected in the following manner. Briefly, blood samples from a patient with a target disease, such as HCV, are analyzed with antibodies to individual HCV polypeptide regions (*e.g.*, HCV core, E1, E2/SNI and NS2-NS5 regions), in order to determine which antigenic fragments are present in the patient's serum. In patients treated with alpha interferon to give temporary remission, some antigenic determinants will disappear and be supplanted by endogenous antibodies to the antigen. Such antigens are useful as immunogenic portions within the context of the present invention (Hayata et al., *Hepatology 13*:1022-1028, 1991; Davis et al., *N. Eng. J. Med. 321*:1501-1506, 1989).

With respect to the treatment of HBV, particularly preferred immunogenic portions for incorporation into nucleic acids include HBeAg, HBcAg, and HBsAgs.

Further, more than one immunogenic portion (as well as immunomodulatory factors, if desired) may be incorporated into the nucleic acid. For example, within one embodiment a nucleic acid may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such constructs may be administered in order to prevent or treat acute and chronic hepatitis infections of either type B or C. Similarly, within other embodiments, a nucleic acid may be prepared which directs the co-expression of both an immunogenic portion of the hepatitis B X antigen, as well as an immunogenic portion of the hepatitis C polypeptide. Such a construct may similarly be administered in order to treat hepatocellular carcinoma that is associated with either hepatitis B or C. In addition, because those individuals chronically infected with hepatitis B and C are at higher risk for developing hepatocellular carcinoma, such a vector may also be utilized as a prophylactic treatment for the disease.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1/K^b transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1/K^b transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med. 173*:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, op. cit., Milich et al., *Proc. Natl. Acad. Sci. USA 85*:1610-1614, 1988; Willis, *Nature 340*:323-324, 1989; Griffiths et al., *J. Virol. 65*:450-456, 1991).

The present invention also includes compositions and methods for treating, as well as vaccines for preventing, various feline diseases, including for example feline leukemia virus ("FeLV") and feline immunodeficiency virus ("FIV") infections. This viruses are discussed more fully in co-pending Application Serial No. 07/948,358.

Feline leukemia virus (FeLV) is a retrovirus of the oncornavirus subfamily. FeLV is presently believed to exist in three subgroups - A, B or C - which are differentiated by their envelope antigens gp70 and p15E. FeLV is also comprised of a number of core antigens, including p15, p12, p27, and p10, which are highly conserved for all subgroups of

FeLV (*see* Geering et al., *Virology 36*:678-680, 1968; Hardy et al., *JAMA 158*:1060-1069, 1971; Hardy et al., *Science 166*:1019-1021, 1969). Within one embodiment of the invention, the nucleic acid directs the expression of at least one portion of a feline leukemia virus antigen selected from the group consisting of p15*gag*, p12*gag*, p27*gag*, p10*gag*, p14*pol*, p80*pol*, p46*pol*, gp70*env*, and p15*env*. Within a particularly preferred embodiment, the nucleic acid directs the expression of gp85*env*. Sequences which encode these antigens may be readily obtained given the disclosure provided herein (*see* Donahue et al., *J. Virol. 62*(3):722-731, 1988; Stewart et al., *J. Virol. 58*(3):825-834, 1986; Kumar et al., *J. Virol. 63*(5):2379-2384, 1989; Elder et al., *J. Virol. 46*(3):871-880, 1983; Berry et al., *J. Virol. 62*(10):3631-3641, 1988; Laprevotte et al., *J. Virol. 50*(3):884-894, 1984).

Feline immunodeficiency virus (FIV) has been classified as a retrovirus of the lentivirus subfamily, based upon the magnesium requirement for reverse transcriptase (RT) and the morphology of viral particles (*see* Pederesen et al., *Science 235*:790-793, 1987). The feline immunodeficiency virus is morphologically and antigenically distinct from other feline retroviruses, including feline leukemia virus, type C oncorna virus (RD-114), and feline syncytium-forming virus (FeSFV) (*see* Yamamoto et al., "Efficacy of experimental FIV vaccines, (Abstract), First International Conference of Feline Immunodeficiency Virus Researchers, University of California, Davis, CA, Sep. 4-7, 1991). Within one embodiment of the invention, the nucleic acid directs the expression of at least one immunogenic portion of an feline immunodeficiency virus antigen selected from the group consisting of p15*gag*, p24*gag*, p10*gag*, p13*pol*, p62*pol*, p15*pol* and p36*pol*. Within a particularly preferred embodiment, the nucleic acid directs the expression of gp68*env*, gp27*env* and *rev*. Within the context of the present invention, "*rev*" is understood to refer to the antigen corresponding to the *rev* open reading frame (*see*, Phillips et al., First International Conference, *supra*). Sequences which encode these antigens may be readily obtained by one of skill in the art given the disclosure provided herein (*see* Phillips et al., *J. Vir. 64*(10):4605-4613, 1990; Olmsted et al., *PNAS 86*:2448-2452, 1989; Talbott et al., *PNAS 86*:5743-5747, 1989).

Still other examples include a nucleic acid which directs the expression of a non-tumorigenic, altered ras (ras*) gene. Briefly, the ras* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras* genes are found in pre-neoplastic tumors, and therefore immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of

the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science 248*:1101-1104, 1990), which, if treated early, may prevent tumorigenesis.

Ras* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas. However, the spectrum of mutations occurring in the ras* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras* occur primarily (*in vivo*) in only 3 codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors.

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered p53 (p53*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells, and thus was initially classified as an oncogene (Linzer and Levine, *Cell 17*:43-52, 1979; Lane and Crawford, *Nature 278*:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol. 63*:739-746, 1989). It now appears that p53 is a tumor suppressor gene, which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles, one through deletion, and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (*e.g.*, $p53^{*1}$, $p53^{*2}$, etc.) are clustered between amino-acid residues 130 to 290 (*see* Levine et al., *Nature 351*:453-456, 1991; *see also* the following references which describe specific mutations in more detail: Baker et al., *Science 244*:217-221, 1989; Nigro et al., *Nature 342*:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature 348*:681-682, 1990; Takahashi et al., *Science 246*:491-494, 1989; Iggo et al., *Lancet 335*:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA 86*:2858-2862, 1989; Mackay et al., *Lancet 11*:1384-1385,1988; Kelman et al., *Blood 74*:2318-2324, 1989; Malkin et al., *Science 250*:1233-1238, 1990; Baker et al., *Cancer Res. 50*:7717-7722,

1991; Chiba et al., *Oncogene 5*:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene 5*:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol. 10*:5502-5509, 1990; Bartek et al., *Oncogene 5*:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA 87*:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA 87*:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA 87*:5863-5867, 1990; and Romano et al., *Oncogene 4*:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature 350*:429-431, 1991) describes specific G to T mutations in codon 249, in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B_1, a liver carcinogen which is known to be a food contaminant in Africa.

Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286. Three "hot spots" of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature 351*:453-456, 1991). These alterations as well as others which are described above result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence ($p53^*$).

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered Rb (Rb^*) gene. Briefly, retinoblastoma is a childhood eye cancer associated with the loss of a gene locus designated Rb, which is located in chromosome band 13q14. A gene from this region has been cloned which produces a nuclear phosphoprotein of about 110 kd (Friend et al., *Nature 323*:643, 1986; Lee et al., *Science 235*:1394, 1987; and Fung et al., *Science 236*:1657, 1987).

Rb is believed to be a negative regulator of cellular proliferation, and has a role in transcriptional control and cell-cycle regulation. Rb binds to at least seven proteins found in the nucleus, and in particular, appears to be involved with a cellular transcription factor which has been designated both E2F (Bagchi et al., *Cell 62*:659-669, 1990) and DRTF (Shivji and La Thangue, *Mol. Cell. Biol. 11*:1686-1695, 1991). Rb is believed to restrict cellular growth by sequestering a variety of nuclear proteins involved in cellular proliferation.

Deletions within the Rb gene have been detected which evidence that the Rb gene may be responsible for tumorigenicity. These deletions include, for example, a

deletion in exon 21 in a prostate cancer and bladder cancer cell line (Bookstein et al., *Science 247*:712-715, 1990; Horowitz et al., *Science 243*:937, 1989), a deletion of exon 16 in a small-cell carcinoma of the lung (Shew et al., *Cell Growth and Diff. 1*:17, 1990), and a deletion between exons 21 and 27 (Shew et al., *Proc. Natl. Acad. Sci. USA 87*:6, 1990). Deletion of these exons results in the production of a protein containing a novel coding sequence at the junction of the deleted exons. This novel protein coding sequence may be used as a marker of tumorigenic cells, and an immune response directed against this novel coding region may eliminate tumorigenic cells containing the Rb exon deletion.

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered gene which causes Wilms' tumor. Briefly, Wilms' tumor is typically found in children younger than 16 years of age. One child in 10,000 will develop this tumor, which comprises approximately 5% of childhood cancers. The tumor usually presents itself as a large abdominal mass which is surrounded by a fibrous pseudocapsule. Approximately 7% of the tumors are multifocal in one kidney, and 5.4% are involved with both kidneys. The Wilms' tumor gene has been localized to chromosome 11p13, and a cDNA clone (wt1) has been isolated that is characteristic of a tumor suppressor gene (Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; and Haber et al., *Cell 61*:1257, 1990). The wt1 gene encodes a protein which contains four zinc fingers and a glutamine and proline rich amino terminus. Such structures are believed to be associated with transcriptional and regulatory functions.

Mutations of the Wilms' tumor gene include the insertion of lysine, threonine, and serine between the third and forth zinc fingers. A wt1 protein which contains such insertions does not bind to the EGR-1 site. A second alternative mutation results in the insertion of about 17 amino acids in the region immediately NH_2-terminal to the zinc finger domain (Madden et al., *Science 253*:1550-1553, 1991; Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; Haber et al., *Cell 61*:1257, 1990; and Buckler et al., *Mol. Cell. Biol. 11*:1707, 1991).

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered mucin. Mucins are large molecular weight glycoproteins which contain approximately 50% carbohydrate. Polymorphic epithelial mucin (PEM) is a tumor-associated mucin (Girling et al., *Int. J. Cancer 43*:1072-1076, 1989) which is found in the serum of cancer patients. The full-length cDNA sequence has been identified (Gendler et al., *J. Biol. Chem. 265*(25):15286-15293, 1990; Lan et al., *J. Biol. Chem. 265*(25):15294-15299, 1990; and Ligtenberg et al., *J. Biol. Chem. 265*:5573-5578, 1990). Breast tumors and pancreatic tumors both express a mucin with an identical core sequence, containing a 20 amino-acid tandem repeat (Jerome et al.,

Cancer Res. 51:2908-2916, 1991). CTL lines which have been developed to breast tumors which cross-react with pancreatic tumor targets, and further appear to specifically recognize the specific 20 amino-acid tandem repeat (Jerome et al., *supra*). A sequence encoding one or more of the 20 amino-acid tandem repeats may be expressed by a nucleic acid of the present invention, in order to develop an immune response against tumor cells which contain this sequence.

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of an altered DCC (deleted in colorectal carcinomas) gene. Briefly, a very common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70% of carcinomas, and in almost 50% of late adenomas. A presumptive tumor suppressor gene (DCC) from this region has been identified (Fearon et al., 1990), which encodes a protein with significant homology to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin (reviewed by Edelman in *Biochem 27*:3533-3543, 1988). This protein is believed to play a role in the development of colorectal tumors, perhaps through alterations in normal cell-cell and/or cell-extracellular matrix interactions.

The DCC gene is expressed in normal colonic mucosa, but its expression is reduced or absent in the majority of colorectal carcinomas (Solomon, *Nature 343*:412-414, 1990). This loss of expression has been associated in some cases with somatic mutations of the DCC gene. A contiguous stretch of DNA comprising 370 kb has been cloned which encodes an approximately 750 amino acid protein (Fearon et al., *Science 247*:49-56, 1990).

Within another embodiment of the present invention, a nucleic acid is provided which directs the expression of MCC (mutated in colorectal cancer) or APC. Both MCC and APC have been identified as tumor suppressor genes (Kinzler et al., *Science 251*:1366-1370, 1991) which undergo mutation in familial adenomatous polyposis (FAP). FAP is believed to be the most common autosomal dominant disease which leads to cancer, and it affects at least 1 in 5,000 individuals in the United States (Nishiho et al., *Science 253*:665-669, 1991). Affected individuals usually develop hundreds to thousands of adenomatous polyps of the colon and rectum, which may progress to carcinoma. Gardner's syndrome ("GS," a variant of FAP) presents desmoid tumors, osteomas, and other neoplasms together with multiple adenomas of the colon and rectum. This proliferation is believed to be induced by loss or inactivation of the familial adenomatous polyposis gene (and in particular, MCC and APC) which is found on chromosome 5q.

For example, in Nishiho et al. (*supra*), the following germ line mutations of the APC gene were found in FAP and GS patients: (1) Codon 280, a serine to stop mutation (in a patient with mandibular osteoma), (2) codon 302, an arginine to stop mutation in two separate patients, one with a desmoid tumor, (3) codon 414, an arginine to

cysteine mutation in a patient with mandibular osteoma, and (5) codon 713, a serine to stop mutation in another patient with mandibular osteoma (Nishiho et al., *Science 253*:665-669, 1991). In addition, six point mutations were identified in MCC codon numbers 12, 145, 267, 490, 506, and 698, as well as an additional 4 somatic mutations in APC (codons number 289, 332, 438, and 1338).

Within other embodiments of the invention, a nucleic acid is provided which directs the expression of an altered receptor which is functionally locked or stuck in an "ON" or "OFF" mode. Briefly, many cellular receptors are involved in cell growth by monitoring the external environment and signaling the cell to respond appropriately. If either the monitoring or signaling mechanisms fail, the cell will no longer respond to the external environment and may exhibit uncontrolled growth. Many different receptors or receptor-like structures may function as altered cellular components, including, for example, *neu* and mutated or altered forms of the thyroid hormone receptor, the PDGF receptor, the insulin receptor, the Interleukin receptors (*e.g.*, IL-1, -2, -3, etc. receptors), or the CSF receptors, such as the G-CSF, GM-CSF, or M-CSF receptors.

For example, *neu* (also referred to as the Human Epidermal Growth Factor Receptor "HER" or the Epidermal Growth Factor "EGF" receptor) is an altered receptor which is found in at least 28% of women with breast cancer. A cDNA clone which encodes this protein has been isolated (Slamon et al., *Science 244*:707-712, 1989; Slamon et al., *Cancer Cells 7*:371-380, 1989; Shih et al., *Nature 290*:261, 1981). This clone encodes a protein that has extracellular, transmembrane, and intracellular domains (Schechter, *Nature 312*:513, 1984; Coussens et al., *Science 230*:1132, 1985) and thus is believed to encode the neu receptor.

Studies of the rat *neu* gene isolated from chemically induced neuroglioblastoma cells indicate that it contains a single mutation at position 664 from valine to glutamic acid (Bargmann et al., *EMBO J. 7*:2043, 1988). In other studies, baby rats which were treated with N-ethyl-N-nitrosourea developed malignant tumors of the nervous system. All 47 trigeminal schwannomas and 12 neurinomas which developed carried a T to A transversion at position 664 of the neu gene (Nikitin et al., *Proc. Natl. Acad. Sci USA 88*:9939-9943, 1991).

Other altered receptors may also be expressed by nucleic acids in order to destroy selected tumor cells. For example, a deletion in chromosome 3p21-p25 has been associated with small-cell lung carcinomas (Leduc et al., *Am. J. Hum. Genet. 44*:282-287, 1989). A deletion is believed to occur in the ERBAb gene which otherwise codes for a DNA-binding thyroid hormone receptor (THR).

Alterations in receptors as described above result in the production of protein(s) (or receptors) containing novel coding sequence(s). The novel protein(s)

encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s).

If the altered cellular component is associated with making the cell tumorigenic, then, it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment, the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced into the gene which encodes the altered cellular component, downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

Within one embodiment, the ras* gene is truncated in order to render the ras* protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of ras* functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the ras* gene is truncated in the purine ring formation, for example around the sequence which encodes amino acid number 110. The ras* gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s) are encoded by the nucleic acid, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

Within another embodiment, the p53* protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations of the p53 protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment, p53* is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both neu and bcr/abl may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above.

It should be noted, however, that if the altered cellular component is only associated with non-tumorigenic cells in general, and is not required or essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-

tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include Rb*, ubiquitin*, and mucin*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays. A particularly preferred method for determining immunogenicity is the CTL assay.

Once a sequence encoding at least one anti-tumor agent has been obtained, it is preferable to ensure that the sequence encodes a non-tumorigenic protein. Various assays are known and may easily be accomplished which assess the tumorigenicity of a particular cellular component. Representative assays include tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

For this and many other aspects of the invention, tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of an anti-tumor agent. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if injected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the nucleic acid is delivered to syngeneic murine cells, followed by administration into nude mice. The mice are visually examined for a period of 2 to 8 weeks after administration in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst. 48*:1531-1533, 1972; Furesz et al., "Tumorigenicity testing of cell lines considered for production of biological drugs," *Abnormal Cells*, New Products and Risk, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std. 13*:135-141, 1985). Tumorigenicity may also be assessed by visualizing colony formation in soft agar (MacPherson and Montagnier, *Vir. 23*:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an anti-tumor agent (*e.g.*, Stewart et al., *Cell 38*:627-637, 1984; Quaife et al., *Cell 48*:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA 86*:5615-5619, 1989). In transgenic animals, the gene of interest may be expressed in all tissues of the animal. This unregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

In addition to tumorigenicity studies, it is generally preferable to determine the toxicity of the toxic palliatives, such as anti-tumor agent(s), prior to administration. A variety of methods well known to those of skill in the art may be utilized to measure such toxicity, including for example, clinical chemistry assays which measure the systemic levels of various proteins and enzymes, as well as blood cell volume and number.

Cell mediated and humoral responses may also be induced against a pathogenic agent, particularly viral and bacterial diseases, by administration of immunogenic portion(s) as discussed above. Briefly, immunogenic portions carrying relevant epitopes can be produced in a number of known ways (Ellis and Gerety, *J. Med. Virol. 31*:54-58, 1990), including chemical synthesis (Bergot et al., *Applied Biosystems Peptide Synthesizer User Bulletin No. 16, 1986*, Applied Biosystems, Foster City, California) and DNA expression in recombinant systems, such as the insect-derived baculovirus system (Doerfler, *Current Topics in Immunology 131*:51-68, 1986), mammalian-derived systems (such as CHO cells) (Berman et al., *J. Virol. 63*:3489-3498, 1989), yeast-derived systems (McAleer et al., *Nature 307*:178-180), and prokaryotic systems (Burrel et al., *Nature 279*:43-47, 1979).

The present invention also provides a nucleic acid capable of immune down-regulation. Specific down-regulation of inappropriate or unwanted immune responses, such as in autoimmune or pseudo-autoimmune diseases such as chronic hepatitis, diabetes, rheumatoid arthritis, graft vs. host disease and Alzheimer's, or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products, or active portion thereof, which suppress surface expression of transplantation (MHC) antigen. Within the present invention, an "active portion" of a gene product is that fragment of the gene product which must be retained for biological activity. Such fragments or active domains can be readily identified by systematically removing nucleotide sequences from the protein sequence, transforming target cells with the resulting recombinant nucleic acid, and determining MHC class I presentation on the surface of cells using FACS analysis or other immunological assays, such as a CTL assay. These fragments are particularly useful when the size of the sequence encoding the entire protein exceeds the capacity of the viral carrier. Alternatively, the active domain of the MHC antigen presentation inhibitor protein can be enzymatically digested and the active portion purified

by biochemical methods. For example, a monoclonal antibody that blocks the active portion of the protein can be used to isolate and purify the active portion of the cleaved protein (Harlow et al., *Antibodies: A Laboratory Manual*, Cold Springs Harbor, 1988).

Within one embodiment, the suppression is effected by specifically inhibiting the activation of display of processed peptides in the context of self MHC molecules along with accessory molecules such as CD8, intercellular adhesion molecule -1 (ICAM-1), ICAM-2, ICAM-3, leukocyte functional antigen-1 (LFA-1) (Altmann et al., *Nature 338*:521, 1989), the B7.1-.3 molecule (Freeman et al., *J. Immunol. 143*:2714, 1989), LFA-3 (Singer, *Science 255*:1671, 1992; Rao, *Crit. Rev. Immunol. 10*:495, 1991), or other cell adhesion molecules. Antigenic peptide presentation in association with MHC class I molecules leads to CTL activation. Transfer and stable integration of specific sequences capable of expressing products expected to inhibit MHC antigen presentation block activation of T-cells, such as $CD8^+$ CTL, and therefore suppress graft rejection. A standard CTL assay is used to detect this response. Components of the antigen presentation pathway include the 45 Kd MHC class I heavy chain, β_2-microglobulin, processing enzymes such as proteases, accessory molecules, chaperonines such as calnexin (Gaczynska, *et al.*, *Nature*, *365*: 264-282, 1993), and transporter proteins such as PSF1, TAP1 and TAP 2 (Driscoll, *et al.*, *Nature*, *365*: 262-263, 1993).

In an alternative example, the recombinant nucleic acid directs the expression of a gene product or an active portion of a gene product capable of binding β_2-microglobulin. Transport of MHC class I molecules to the cell surface for antigen presentation requires association with β_2-microglobulin. Thus, proteins that bind β_2-microglobulin and inhibit its association with MHC class I indirectly inhibit MHC class I antigen presentation. Suitable proteins include the H301 gene product. Briefly, the H301 gene, obtained from the human cytomegalovirus (CMV) encodes a glycoprotein with sequence homology to the β_2-microglobulin binding site on the heavy chain of the MHC class I molecule (Browne et al., *Nature 347*:770, 1990). H301 binds β_2-microglobulin, thereby preventing the maturation of MHC class I molecules, and renders transformed cells unrecognizable by cytotoxic T-cells, thus evading MHC class I restricted immune surveillance.

Within another embodiment, the recombinant nucleic acid directs the expression of a protein or active portion of a protein that binds to newly synthesized MHC class I molecules intracellularly. This binding prevents migration of the MHC class I molecule from the endoplasmic reticulum, resulting in the inhibition of terminal glycosylation. This blocks transport of these molecules to the cell surface and prevents cell recognition and lysis by CTL. For instance, one of the products of the E3 gene may be used to inhibit transport of MHC class I molecules to the surface of the transformed cell.

More specifically, E3 encodes a 19 kD transmembrane glycoprotein, E3/19K, transcribed from the E3 region of the adenovirus 2 genome. Within the context of the present invention, tissue cells are transformed with a recombinant nucleic acid containing the E3/19K sequence, which upon expression produces the E3/19K protein. The E3/19K protein inhibits the surface expression of MHC class I surface molecules, and cells transformed by the nucleic acid evade an immune response. Consequently, donor cells can be transplanted with reduced risk of graft rejection and may require only a minimal immunosuppressive regimen for the transplant patient. This allows an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

Another alternative method of immunosuppression involves the use of antisense message, ribozyme, or other gene expression inhibitor specific for T-cell clones which are autoreactive in nature. These block the expression of the T-cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using a viral vector delivery system.

Other proteins, not discussed above, that function to inhibit, suppress or down-regulate MHC class I antigen presentation may also be identified and utilized within the context of the present invention. In order to identify such proteins, in particular those derived from mammalian pathogens (and, in turn, active portions thereof), a recombinant nucleic acid that expresses a protein or an active portion thereof suspected of being capable of inhibiting MHC class I antigen presentation is transformed into a tester cell line, such as BC. The tester cell lines with and without the sequence encoding the candidate protein are compared to stimulators and/or targets in the CTL assay. A decrease in cell lysis corresponding to the transformed tester cell indicates that the candidate protein is capable of inhibiting MHC presentation.

Another alternative method to determine down-regulation of MHC class I surface expression is by FACS analysis. More specifically, cell lines are transformed with a recombinant nucleic acid encoding the candidate protein. After drug selection and expansion, the cells are analyzed by FACS for MHC class I expression and compared to that of non-transformed cells. A decrease in cell surface expression of MHC class I indicates that the candidate protein is capable of inhibiting MHC presentation. This aspect of the present invention is further discussed in co-pending application Serial No. 08/116,827.

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible

cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by utilizing nucleic acids that produce, *in vivo*, an analogue to either of the partners in an interaction. Such an analogue is known as a blocking agent.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a retroviral nucleic acid carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

For example, in the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a nucleic acid expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a retroviral vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env may also be constructed. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., *J. Virol. 62*:139, 1988; Fisher et al., *Science 233*:655, 1986).

Another aspect of the invention involves the delivery of suppressor genes which, when deleted, mutated or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

Sequences which encode the above-described altered cellular components may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC

No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (*see, for example*, ATCC No. 41001 which contains a sequence which encodes the normal ras protein, ATCC No. 57103 which encodes abl; and ATCC Nos. 59120 or 59121 which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (*see* Sambrook et al., *supra.*, 15.3 *et seq.*). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

Other nucleic acid molecules that encode the above-described substances, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including for example depositories such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1b), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Molecularly cloned genomes which encode the hepatitis B virus may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG 5*(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA 78*:2606-2610, 1981). (Note that correctable errors occur in the sequence of ATCC No. 45020.)

Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed

with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Nucleic acid molecules that are suitable for use with the present invention may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, California).

ADMINISTRATION

In aspects of the invention the nucleic acids condensed with a nucleic acid condensing agent are provided in a pharmaceutically acceptable carrier. Such compositions may comprise buffers such as physiologically-buffered saline, phosphate-buffered saline, and the like, carbohydrates, such as glucose, mannose, sucrose or mannitol, proteins, polypeptides or amino acids, such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide) and preservatives. In addition, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as, for example, cytokines like β-interferon.

Compositions of the present invention may be formulated for the manner of administration indicated, including for example, for oral, nasal, venous, intracranial, intraperitoneal, subcutaneous, or intramuscular administration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

CHEMICAL MODIFICATION OF POLYCATION WITH PEG

Methoxypolyethylene glycol (PEG) *N*-hydroxysuccinimidyl-glutarate is prepared by first conjugating glutarate to the free hydroxy terminus of PEG and then conjugating an N-hydroxysuccinimide in two steps. Adding a glutarate group is done following the procedure of Joppich and Luisi (*Macromol. Chem. 180*:1381, 1979). Fifty g of PEG are dissolved in 80 ml of toluene and distilled. After cooling, the solution is reacted with glutaric anhydride (11.5 g) in dicycloethane (200 ml) and dry pyridine (5 ml). The mixture is refluxed under nitrogen gas for 3 days, filtered and the solvent evaporated. The residue is dissolved in 100 ml of water and washed twice with 50 ml of diethyl ether. PEG-glutarate is then extracted with two 50 ml chloroform washes, and the residual chloroform is evaporated. Approximately 38 g of PEG-glutarate is obtained.

Next, NHS is attached to the glutarate (Anderson et al., *J. Am. Chem. Soc. 86*:1839, 1964). PEG-glutarate is dissolved in 200 ml of dimethylformamide at 37°C. 0.94 g of *N*-hydroxysuccinimide and 1.7 g of dicyclohexylcarbodiimide in dry dimethylformamide are added and the mixture stirred vigorously in an ice bath, followed by stirring at room temperature for 24 h. Precooled benzene (100 ml) is added, and methoxypolyethylene glycol *N*-hydroxysuccinimidyl glutarate is precipitated by the dropwise addition of 200 ml of petroleum ether at 0°C. The precipitate is collected on a sintered glass filter. NHS-PEG is reprecipitated three times. NHS-PEG may be stored in a desiccator at -20°C until used.

NHS-PEG is conjugated to a free amino group of polylysine at ratios of 2-30 in 0.1 M PBS (pH 7.5). The solution is stirred for 30 min at room temperature, and any remaining activated ester is removed by reaction with excess ε-aminocaproic acid for 5 min. Unbound NHS-PEG may be removed from the reaction mixture by molecular-exclusion chromatography on P-100 (Bio-Rad) in 0.1 M PBS (pH 7.5). The attachment of PEG to polylysine may be confirmed by SDS-PAGE. The degree of amino group modification in the nucleic acid condensing agent is determined by measuring the number of free amino groups with TNBS (Synder and Sobocinski, *Anal. Biochem. 126*:433, 1982) or by NMR spectroscopy against standard PEG preparations.

Example 2

CHEMICAL MODIFICATION OF POLYCATION WITH DEXTRANS

Oxidized dextran, which refers to periodate-oxidized dextran molecules containing multiple aldehyde functions, is prepared by oxidation with $NaIO_4$ (Bobb, H., *Adv. Carbohydr. Chem. 11*:1, 1956). Briefly, 30 g of dextran, M_r 6000 ± 500, is dissolved

in 1 liter of 10 mM sodium borate buffer, containing 0.35 M $NaIO_4$, pH 3.0. After 24 h in the dark, the reaction is terminated by adding a 2-fold molar excess of ethylene glycol for an additional 2 h. The reaction mixture is dialyzed extensively against distilled water and lyophilized. Yields (w/w) of oxidized dextran are typically 45% of starting material. The relative aldehyde content of oxidized dextran may be determined by anthrone assay (Fagnani et al., *Cancer Res. 50*:3638, 1990). Additionally, oxidized dextran may be analyzed for integrity by HPLC.

A solution of oxidized dextran in 100 mM sodium phosphate buffer, pH 7.0, is added in stoichiometric molar ratios from about 1:500 to 1:1500 in two-fold decreasing molar increments to a solution of polylysine (5 mg/ml) in the same buffer. A solution of $NaBH_3CN$ in the same buffer is then added in stoichiometric molar ratios of 1 to 5 relative to dextran, and the solution stirred at 22°C for 2 h. At the end of the incubation, dextran-modified polylysine is separated from unreacted dextran by gel filtration. The level of dextran substitution of polylysine may be determined with the anthrone assay as described below. Unreacted aldehyde groups on dextran bound to polylysine is then reduced by dropwise addition of $NaBH_4$, in 0.05 N NaOH at a molar ratio of 25 to 1 relative to the glucose content of dextrose (33 glucose subunits per M_r 6000 dextran). After incubation for 24 h at 4°C, completion of the reaction may be established by anthrone determination. Dextran-modified polylysine is then extensively dialyzed against 100 mM sodium phosphate, pH 7.0, and stored in this buffer at 4°C.

To determine the extent of dextran conjugation, a standard curve is constructed by adding increasing amounts of activated dextran (10 to 100 µg) to 1 ml of sodium phosphate buffer, pH 7.0, in clean glass tubes in duplicate. To this volume, 3 ml of a freshly made anthrone solution (0.2% in 80% H_2SO_4) are added rapidly and tubes are mixed on a vortex mixer. Tubes then are transferred immediately to a boiling water bath for 15 minutes, and then cooled in an ice water bath for an additional 15 minutes. Color development is determined by reading the absorbance at 625 nm. in a spectrophotometer. With this assay, non-oxidized dextran or oxidized dextran which has been reduced with $NaBH_4$ does not produce any color. Fifty to 100 µg of either unmodified polycation or polycation dextran adducts then are similarly tested, and their absorbance at 625 nm. is plotted against the standard curve prepared from the previous activated dextran standard spectrophotometric determinations. The amount of activated dextran bound to a polycation can be determined from the curve and the molar concentration then is calculated. The concentration of polycation can be determined easily by reading the absorbance at 280 nm. The molar substitution of dextran (m.w. 6,000) per mole of polycation then can be calculated according to the following formula:

$$\frac{\text{MW of specific polycation - nucleic acid complex}}{6{,}000} \times \frac{[\text{Dextran}]\ (\text{mg/ml})}{[\text{Polycation}]\ (\text{mg/ml})}$$

Example 3

ASSAYS FOR DNA CONDENSATION

A number of assays may be used to detect the condensation of the DNA. These assays are generally based on the resulting changes in the physical properties of DNA after condensation. For example, the ability of DNA to bind ethidium bromide is reduced, and the mobility of DNA molecules during electrophoresis in an agarose gel is retarded.

An ethidium bromide dye assay is performed by mixing 10 μg pGL2 control plasmid DNA (Promega Corporation, part No. E1611) in 200 μl condensation buffer (20 mM Hepes/150 mM NaCl, pH 7.4) with candidate condensing agents. Different amounts of candidate condensing agents are added (*e.g.*, 10, 50, 100, 200 or 500 μg). The condensation reactions are incubated at room temperature for 30 minutes, at which time ethidium bromide is added to each reaction to a final concentration of 1 μg/ml. After 15 min, reaction mixtures are transferred into polystyrene tubes or other UV-transparent material and placed on a 260 nm transilluminator. The fluorescent light emission from the DNA-ethidium bromide complex in each reaction mixture is recorded with a camera equipped with a UV filter. The ability of a condensing agent to condense DNA is inversely proportional to the intensity of the fluorescent in each reaction mixture.

A band shift assay is based on the size difference between condensed and non-condensed DNA. Mobility of condensed DNA in an electric field is reduced and can be monitored by agarose gel electrophoresis. The procedure according to Wagner et al. (*Proc. Natl. Acad. Sci. USA 87:*3410, 1990) may be followed. Briefly, lambda DNA is digested with *Eco*RI and *Hind*III restriction enzymes to generate DNA fragments with a size ranging from 564bp to 21.2kb. These fragments are radioactively labeled by filling in the cohesive ends with [^{32}P]dNTPs using polI (Klenow fragment) (Sambrook et al., *Molecular Cloning-A Laboratory Manual*, 1989). Radioactively labeled DNA fragments (35 ng) are mixed with 10, 20, 50, 75, 100, 200, 500 or 1000 ng of the candidate condensing agents in 10 mM Hepes/200 mM NaCl, pH 7.9 at a final volume of 12 μl. After incubating at room temperature for 30 minutes, the samples are electrophoresed on a 1.0% agarose gel with 1X TAE electrophoresis buffer (40 mM Tris acetate/1 mM EDTA, pH 8.0). The gel is dried and autoradiographed for 3 hours at -80°C with XAR film

(Kodak). The ability of the candidate agents to condense DNA is determined by the minimal amount of agent required to retard the mobility of the DNA fragments in the agarose gel. For example, 100 ng of polylysine is able to retard the mobility of the DNA fragments under these conditions. Any condensing agent that retards the mobility of the DNA fragments with less than 100 ng is considered a better DNA condensing reagent than polylysine. Any agents that condense the DNA fragments using less than 200 ng are chosen for further analysis in assays measuring gene transfer efficiency.

Alternatively, 10 μg of unlabeled pGL2 plasmid DNA in 200 ul condensation buffer (20 mM Hepes/150 mM NaCl, pH 7.4) is mixed with various amounts of candidate condensing agents at similar ratios to above. The condensation reactions are incubated at room temperature for 30 minutes, at which time a fraction (1/10 of the total volume) of the samples are electrophoresed on a 0.8% agarose gel which is then stained with ethidium bromide (1 μg/ml) for 30 minutes and washed with water for 30 minutes. The DNA bands are visualized on a transilluminator and photographed. Condensation of plasmid DNA is associated with a decrease in mobility during electrophoresis.

Example 4

EFFICIENCY OF GENE TRANSFER IN VITRO

Polylysine with an average length of 270 amino acid residues is used as a standard in a gene transfer assay. Condensing agents that mediate gene transfer with an efficiency of 50% that of polylysine are chosen for further analysis. Efficiency of polylysine-mediated gene transfer is determined as follows. 3 μg of the luciferase reporter gene construct, pGL2-control, in 250 μl of 20 mM Hepes/150 mM NaCl, pH 7.4 and 10 μg polylysine (Sigma, Cat. #2636) in 250 μl of 20 mM Hepes/150 mM NaCl, pH 7.4, are mixed and incubated at room temperature for 30 minutes, at which time 1 ml of DMEM/2% FBS (fetal bovine serum) is added. The mixture is added to target cells (*e.g.*, HT1080, K562, or other primary cell lines), grown to about 60-80% confluency in 60 mm dishes. After 1 hour at 37°C, 4 ml of DMEM/10% FCS is added and incubation continued at 37°C in a CO_2 incubator for 24 hours. At this time, cells are harvested by scraping the dishes. Reagents for the luciferase assay are commercially available (*e.g.*, Promega Corporation, part No. E1500). Procedures suggested by the manufacturer are followed for the assay. Briefly, harvested cells are lysed. The lysate is then mixed with Luciferase Assay Reagent containing luciferin, ATP, CoA, DTT, EDTA, $MgSO_4$, and Tricine. Luciferase present in the lysate catalyzes a reaction that leads to emission of light. The efficiency of gene transfer is gauged by measuring the intensity of light emission from the mixture with a luminometer.

Example 5

DETERMINATION OF IMMUNOGENICITY

The immunogenicity of modified polylysine and nucleic acid condensed complexes is measured in comparison to unmodified polylysine nucleic acid condensed complexes. These complexes are administered to mice, rabbits, or primates and the extent of the immune response directed against the injected complexes is measured (A) by ELISA and, if antibodies are present, (B) by neutralization of gene transfer efficiency in tissue culture. Experiments in this example are performed in mice owing to the simplicity and ease of this animal model.

An appropriate dose of unmodified polylysine/DNA to achieve measurable antibody levels is first determined. Groups of three mice, in a common strain such as Balb/c, are injected with either 1, 5, 25, or 125 μg of reporter gene construct DNA, pGL2-control complexed with polylysine as in Example 4. Briefly, 20 μg of unmodified polylysine (as a control) are mixed per 6 μg of DNA. The reactions are incubated at room temperature for 30 minutes to allow condensation to occur. This mixture is diluted in HEPES/NaCl buffer. It may be rapidly concentrated in ultrafiltration units, Centriprep-10 or 30 (Amicon) if necessary. The mixture is then administered into an adult mouse by i.v., i.p., s.c., or i.m. injection. A total of 3 or more injections are administered to each mouse, with two to four weeks between each injection. At 1, 2, 3, 4, and 8 weeks after the final injection, blood samples are collected from each mouse. From each individual mouse, antibody titers against polylysine are measured in a standard ELISA assay (Engvall E., *Meth. Enzymol. 70*:419-39, 1980). Briefly, polylysine/DNA complex is used to coat the plates, and dilutions of serum samples are then added. Plates are washed to remove unbound serum antibodies. An anti-mouse immunoglobulin antibody conjugated with a reporter molecule, such as horseradish peroxidase (Tago), is added to each well. A colorimetric determination of the titer of the anti-polylysine/DNA complex antibody is then established. The experiment to determine the effects of modifications on immunogenicity is performed at the lowest convenient does of plasmid DNA/polylysine complex which in the majority of animals give a measurable antibody induction response by the third bleed.

Groups of five mice are then injected with unmodified or modified polylysine/DNA complexes prepared as described above, at the immunogenically appropriate dose and injection schedule determined in the dose response experiment. Mice are bled weekly for three weeks. Sera are separated following clotting at room temperature and tested individually at 1:100 to 1:10,000 dilutions in an ELISA. Titers are determined as the reciprocal of the dilution capable of generating 50% of the maximum response of

each tested nucleic acid complex. The conjugated polylysine-condensed DNA complex will meet the defined criteria for low immunogenicity if the antibody titer is reduced at least three fold and preferably ten fold relative to the titer determined for unconjugated polylysine/DNA complex at the two or the three week bleed time point.

In addition, if the criteria in the ELISA for low immunogenicity are not met, then these same sera that contain antibodies to the complex, either fresh or stored frozen at -80°C, are tested for neutralizing antibodies in the following assay. A reporter plasmid, such as pSV β-Galactosidase (Promega Cat. No. E1081), which expresses β-*gal* is condensed with modified or unmodified polylysine condensing agent under conditions that give efficient gene transfer as described in Example 4. The complex is treated with heat-inactivated FBS (as a control), or heat-inactivated mouse sera from the post injection bleeds of the modified or unmodified polylysine DNA complex injected mice respectively. Transfection efficiency is then tested on a tissue culture target cell such as HT1080 human fibroblasts (ATCC No. CCL121).

Heat-inactivated serum samples are prepared by heating for 30 minutes at 56°C. Quantities of β-*gal* plasmid DNA are reacted with the condensing agent such that approximately 1×10^5 BCFU/ml are generated following a standard transfection onto HT1080 cells. Complexes are mixed 1:10 with heat-inactivated FBS, or heat-inactivated sera from mice previously injected with the same gene transfer complexes and incubated at 37°C for 30 minutes. Transfection efficiency is determined by a standard BCFU assay (*Current Protocols in Molecular Biology, supra*). If the sera contains neutralizing antibodies, the number of BCFU is reduced relative to treatment with heat-inactivated FBS (control). If the serum component is heat-labile, the number of BCFU following treatment with heat-inactivated sera will not deviate significantly from the control. "Percentage survival" is the percentage of BCFU/ml obtained from transfection of nucleic acid/condensing agent complex following treatment with heat-inactivated sera isolated from immune animals (i.e., animals injected with the same nucleic acid/condensing agent) relative to the number of BCFU/ml obtained following treatment with heat-inactivated FBS treatment. The GL2/conjugated-polylysine complex will meet the criteria for low immunogenicity if its percentage survival is at least three-fold, and preferably ten-fold, higher than the percentage survival for the GL2/unconjugated-polylysine complex.

Example 6

COMPLEMENT INACTIVATION ASSAY

A reporter plasmid, such as pSV-β-Galactosidase (Promega Cat. No. E1081), which expresses β-*gal* is condensed with condensing agent under conditions that

give efficient gene transfer as described in Example 4. The complex is treated with heat-inactivated FBS (as a control), human sera, or heat-inactivated human sera. Transfection efficiency is then tested on a tissue culture target cell such as HT1080 human fibroblasts (ATCC No. CCL121).

The assay for serum inactivation of the complex is performed as follows. Blood is drawn from at least two human volunteers. Approximately 20-70 mL of blood is collected and allowed to clot for 20-30 minutes at room temperature. Blood samples are centrifuged at 2000 x g for 10 minutes at 4°C. Serum is frozen in approximately 1.0 mL aliquots and stored at -80°C. Vials from each batch were tested for total classical complement activity (Quantiplate™, Kallestad Labs, Inc., Chaska, MN), and only batches with normal (CH_{100} unit activity at least 60) levels of complement activity were used. Heat-inactivated serum samples are prepared by heating for 30 minutes at 56°C. Quantities of β-gal plasmid DNA are condensed with the condensing agent such that 10^4 to 10^5 BCFU/ml are generated following a standard transfection onto HT1080 cells). Complexes are mixed 1:10 with heat-inactivated FBS, human sera, or human heat-inactivated sera and incubated at 37°C for 30 minutes. Transfection efficiency is determined by a standard BCFU assay (*Current Protocols in Molecular Biology, supra*). If a human serum component inactivates the complex, the number of BCFU is reduced relative to treatment with heat-inactivated FBS (control). If the serum component is heat-labile, the number of BCFU following treatment with heat-inactivated human sera will not deviate significantly from the control. A condensing agent with reduced sensitivity to human serum inactivation is defined as an increase of BCFU of two-fold or greater relative to polylysine-condensed DNA.

From the foregoing, it will be evident that although specific embodiments of the invention have been described herein for the purposes of illustrating the invention, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

1. Nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with polyalkylene glycol, wherein the nucleic acid condensing agent in combination with nucleic acids exhibits low immunogenicity.

2. The nucleic acids of claim 1 wherein the polyalkylene glycol is polyethylene glycol.

3. The nucleic acids of claim 2 wherein the polyethylene glycol has a molecular weight ranging from 200 to 10,000.

4. The nucleic acids of claim 1 wherein the polycation is selected from the group consisting of polylysine, protamines, spermine, spermidine, polyornithine, polyarginine and putrescine.

5. The nucleic acids of claim 1, further comprising a pharmaceutically acceptable carrier.

6. The nucleic acids of claim 1, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.

7. The nucleic acids of claim 6 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

8. Nucleic acids condensed with a nucleic acid condensing agent comprising a polycation chemically conjugated with a polysaccharide, wherein the nucleic acid condensing agent in combination with nucleic acids exhibits low immunogenicity.

9. The nucleic acids of claim 8 wherein the polysaccharide is dextran.

10. The nucleic acids of claim 9 wherein the dextran has a molecular weight ranging from 1,000 to 90,000.

11. The nucleic acids of claim 8 wherein the polycation is selected from the group consisting of polylysine, protamines, spermine, spermidine, polyornithine, polyarginine and putrescine.

12. The nucleic acids of claim 8, further comprising a pharmaceutically acceptable carrier.

13. The nucleic acids of claim 8, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.

14. The nucleic acids of claim 13 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

15. A gene delivery vehicle chemically conjugated with polyalkylene glycol, the conjugated product exhibiting low immunogenicity.

16. The gene delivery vehicle of claim 15 wherein the polyalkylene glycol is polyethylene glycol.

17. The gene delivery vehicle of claim 16 wherein the polyethylene glycol has a molecular weight ranging from 200 to 10,000.

18. The gene delivery vehicle of claim 15, further comprising a pharmaceutically acceptable carrier.

19. The gene delivery vehicle of claim 15, further comprising a ligand capable of targeting the gene delivery vehicle to a selected cell type.

20. The gene delivery vehicle of claim 19 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor, and erythropoietin.

21. A gene delivery vehicle chemically conjugated with polysaccharide, the conjugated product exhibiting low immunogenicity.

22. The gene delivery vehicle of claim 21 wherein the polysaccharide is dextran.

23. The gene delivery vehicle of claim 22 wherein the dextran has a molecular weight ranging from 1,000 to 90,000.

24. The gene delivery vehicle of claim 22, further comprising a pharmaceutically acceptable carrier.

25. The gene delivery vehicle of claim 22, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.

26. The gene delivery vehicle of claim 25 wherein the ligand is selected from the group consisting of transferrin, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

27. Nucleic acids condensed with a nucleic acid condensing agent comprising a basic amino acid region of at least 50 amino acids, wherein the region has at least 40% basic amino acids, less than 5% acidic amino acids and a predicted isoelectric point of at least 9, wherein the nucleic acid condensing agent in combination with nucleic acids exhibits low immunogenicity.

28. The nucleic acids of claim 27 wherein the basic amino acid region is derived from a protein selected from the group consisting of histones and protamines.

29. The nucleic acids of claim 27 wherein there are a plurality of basic amino acid regions in a tandem array.

30. The nucleic acids of claim 29 wherein the array contains 1-10 basic amino acid regions.

31. The nucleic acids of claim 27, further comprising a pharmaceutically acceptable carrier.

32. The nucleic acids of claim 27, further comprising a ligand capable of targeting the nucleic acids to a selected cell type.

33. The nucleic acids of claim 32 wherein the ligand is selected from the group consisting of transferring, asialoglycoprotein, antibody, antibody fragments, low density lipoproteins, interleukins, GM-CSF, G-CSF, M-CSF, stem cell factor and erythropoietin.

34. A method of gene transfer in a patient comprising administering the nucleic acids according to any one of claims 1, 8, 14, 20, or 25 in a pharmaceutically acceptable carrier to a patient.

35. The method according to claim 34, further comprising adding a ligand to the nucleic acid condensing agent which is capable of targeting the nucleic acids to a selected cell type.

36. The method according to claim 34 wherein the nucleic acids stimulate an immune response upon administration to the patient.

37. The method according to claim 34 wherein the nucleic acids suppress an immune response.

38. The method according to claim 34 wherein the nucleic acids encode a prodrug.

39. The method according to claim 34 wherein the nucleic acids encode a cytokine.

40. The method according to claim 34 wherein the nucleic acids are administered to a tumor.

41. The method according to claim 34 wherein the nucleic acids are capable of constitutive production of protein.